Hypericum perforatum subsp. *angustifolium*: study of xanthone biosynthesis *in planta* and in *in vitro* systems

Dissertation

submitted to and approved by the

Department of Life Sciences
University of Braunschweig, Institut of Pharmaceutical Biology
and the
Faculty of Mathematics, Physics and Natural Sciences
University of Rome

In candidancy for the degree of a
Doktorin der Naturwissenschaften (Dr. Rer. Nat.)/
Dottore di Ricerca in Scienze Botaniche

by
Noemi Tocci
from Tivoli (Rome), Italy

Submitted on **16 December 2012**

Oral examination on **21 February 2013**

Professorial advisors: Prof Gabriella Pasqua
Prof. Ludger Beerhues,

2013

*) Either the German or the Italian form of the title may be used

INDEX

Chapter 1:

Introduction

1.1 The genus *Hypericum*

The genus *Hypericum*, Hypericaceae family, previously classified as Guttiferae or Clusiaceae (Stevens 2007), shares with all the others genera of the family peculiar characteristics: entire opposite leaves, polypetalous corolla, stamens grouped in bundles, seeds without endosperm and presence of glandular secretions. It includes about 450 species of trees (e.g. *H. bequaertii*), bushes and herbs spread all over the temperate areas of the World. The genus is not present in extremely dry, hot or cold habitats. The name of the genus *Hypericum* probably originates from the two Greek words: *Hyper* = over and *eikon* = image, which indicates that plants of this genus were believed to ward off evil or bad luck. The genus *Hypericum* includes about 450 species of flowering plants and represents approximately 80% of the diversity of the family Hypericaceae. The most known and investigated species of the genus is *Hypericum perforatum* which is a medicinal plant used by the popular medicine since centuries.

1.1a *Hypericum perforatum*

Hypericum perforatum (Fig.1) is a completely glabrous herbaceus plant species, characterized by a stem lignified at the base, opposite shortly petiolate or entire lanceolate sessile leaves, yellow flowers made of 5 embricated sepals usually grouped in a terminal cyme; numerous stamens free or united at the base into three or five bundles; 3-5 styles; superior and ovoidal ovary; fruits represented by septicid capsule; cylindric and numerous seeds with reduced or absent endosperm (Bombardelli-Morazzoni, 1995). The leaves are yellow-green in color, with transparent dots throughout the tissue with a few black dots on the lower surface.

Leaves exhibit obvious translucent dots when held up to the light, giving them a 'perforated' appearance, hence the plant's Latin name.

There are morphologic, geographic and cytologic evidences indicating that *H. perforatum* originated from hybridization of two strongly correlated species. One of these is the *H. maculatum* subsp. *immaculatum* (2n=16), which is diffused in Western Europe and in Siberia and morphologically similar to the *H. perforatum*; the other species seems to be the *H. attenuatum* (2n=16), which is diffused from the Siberia to Korea and China and shows the morphological characteristic of *H. perforatum* missing in *H. maculatum*. The hybridization area seems to be the Siberia (Crockett and Robson, 2011).

H. perforatum shows a strong intraspecific variability impossible to classify. According to the Flora of Italy by Pignatti (1982), three different varieties or subspecies are considered: the subspecies *perforatum,* characterized by the distribution of black nodules on the leaf border; the subspecies *veronense* characterized by dark lines on the stem and the subspecies *angustifolium,* rare, with linear lanceolate leaves of 3-7 x 12-25 mm. The anatomic peculiarity of *Hypericum perforatum* is the presence on leaves, stem and flowers of both translucent glands and black globules, secretory structures storing pharmacologically active compounds.

The translucent glands are distributed all over the leaf lamina and they accumulate essential oils (Mathis and Ourrison, 1963), while the dark globules are generally localized on the stem, on the leaf and flower border and on the anthers and they contain hypericin and its derivatives (Baroni Fornasiero et al.,1998).

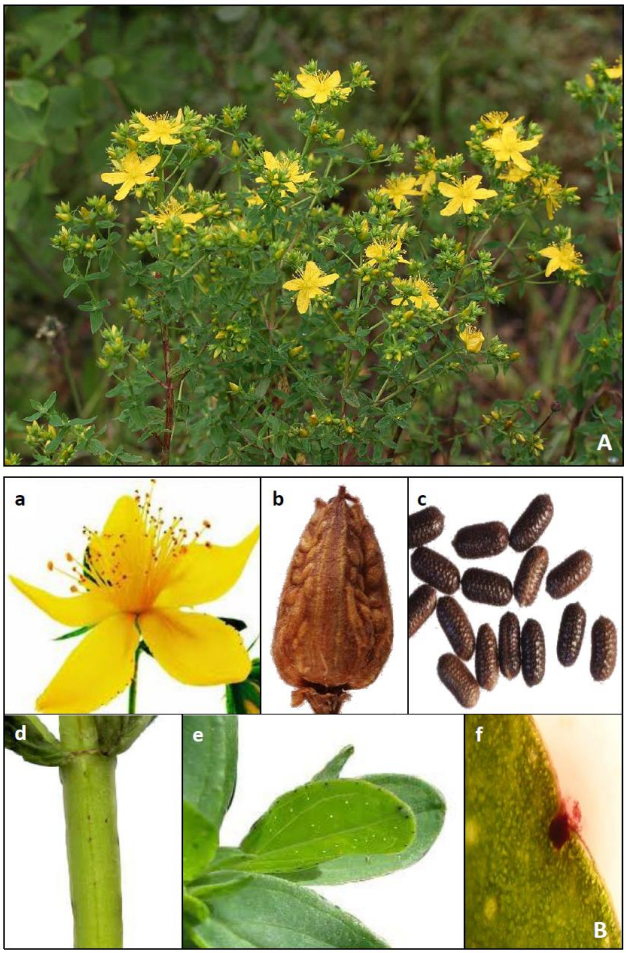


Fig. 1 A, *Hypericum perforatum* ; B, morphological characteristics of the species: Ba, flower with numerous stamens, Bb, capsules, Bc, seeds, Bd, stem with black globules, Be, leaves showing translucent glands and black nodules on the margins, Bf, black globule containing red coloured hypericin.

1.2 Use, Pharmacological Activity and Clinical properties

The use of *H. perforatum* L., commonly known as St. John's wort, has a long tradition in the folk medicine of the Occidental culture. From the time of the ancient Greeks down through the Middle Ages, the plant was believed to possess magical powers and was used to ward off evil and protect against disease. As a practical folk-remedy, it has been widely used for the treatment of cold, chest congestion, menstrual cramps, wounds, burns, snake bites, asthma, tuberculosis, skin problems, kidney troubles and to alleviate nervous disorders.

Nowadays *H. perforatum* is considered a medicinal plant of great interest mainly used in the treatment of mild to moderate depression. *H. perforatum* based products are commercially available as tablets, capsules, oils, spray, tea and tincture form. All the preparations are available as aqueous-alcoholic extracts and oil extracts. The two types of preparations differ for the chemical composition and the therapeutic indication. The major active constituents of the aqueous-alcoholic extracts are phloroglucinols (hyperforins) and flavonoids, together with lesser amounts of hypericins and traces of xanthones and proanthocyanins. This type of preparation is used orally for the treatment of neurological disorders (mild to moderate depression, anxiety, restlessness, nervous tension, migraine, headache), to argue rheumatism, gout and as antidiarrheal and diuretic agents; or topically for the treatment of bites, bruises, wounds and swellings. The major active constituents of the oil extracts are phloroglucinols, flavonoids and essential oils. This type of preparation is used orally for treating gastric

disorders, bacterial infections, mouth and throat pain; or topically in the treatment of wounds and burns (McCutcheon, 2002).

1.3 Phytochemical profile of *Hypericum perforatum*

H. perforatum contains several classes of bioactive compounds. The first phytochemical studies on *H. perforatum* dates back on 1830, when Buchner discovered and isolated the "Red Hypericum" from the plant. Nowadays *H. perforatum* represents the most investigated species among the medicinal plants, because of its documented antidepressive, antitumoral and antimicrobial activities. The most important bioactive compounds of *H. perforatum* can be grouped in 8 classes:

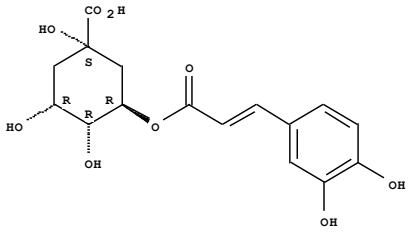
- **Phenylpropans** detected as derivatives of esters of hydroxycinnamic acids as the p-coumaric acid, the caffeic acid and the chlorogenic acid (Fig.2).
- **Flavonoids and flavonoid glucosides**: The flavonoid glucosides having quercetin as aglycone represent the main group (2-4 %) in *H. perforatum*. Generally in the extracts it's possible to detect only a small amount of free quercetin (Fig.2) and this can be maybe due to the hydrolysis of the glucosides during the extraction process. Hyperoside and rutin are generally the major compounds, followed by the isoquercetin and the quercetrin (Fig.2). Kaempferol is not always detected in the extract.
- **Biflavons**: the I3,II8-biapigenin and the amentoflavon are the only detected compounds belonging to this group and found exclusively in the buds and in the blooms.

- **Tannins and proanthocyanidins**: the presence of condensed tannins was demonstrated as early as 1925 by Podoko and described in detail in the following decades. The proanthocyanidins represent 12% of the dry weight (Melzer et al., 1991). The mixture of proanthocyanidins contains only derivatives of catechin and epicatechin of (dimers, trimers and polymers of high molecular weight).
- **Naphtodianthrones**: The emodin anthrone is the precursor of hypericins (Lavie et al. 1995). Pseudohypericin is the main naphtodianthrone generally present in concentration 2 or 4 times higher than hypericin (Fig.2). Among the subspecies of *H. perforatum, H. perforatum* subsp. *angustifolium* was found to be the richer in hypericin (Males et al., 2006). Hypericin is accumulated in the black globules, which are present in all the aerial parts of the plant (flowers, capsules, leaves, stems), but not in the roots.
- **Phloroglucinols:** The hyperforin (Fig.2) and adhyperforin are the main compounds belonging to this class and found in *H. perforatum*. Both the products are accumulated in the reproductive structures (flowers and fruits) (Soelberg et al., 2007). Chatterijee et al. (1998) suggested that hyperforins are the major compounds involved in the antidepressive activity.
- **Xanthones**: xantones are phenolic compounds deriving from the benzoic acids metabolism (Fig.2). This class of molecules is better described in section 1.5.

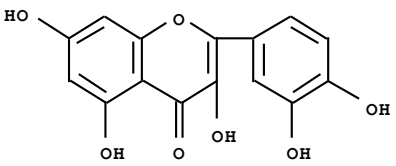
- **Essential oils**: leaves and flowers of *H. perforatum* are characterized by the presence of schizogenous secretory structures containing essential oils. Monoterpens α-pinene and β-pinene, myrcen and limonene and some sesquiterpens are typical costituents of the oil.

1.4 Plant type III Polyketide Synthases (PKSs III)

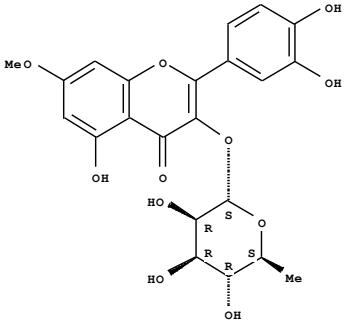
The plant type III Polyketide Synthases are a class of enzymes catalyzing the formation of very important natural products exhibiting a wide variety of bioactivities such as: antibiotic, anticancer, antifungal, immunosuppressant and insecticides (Fig.3). PKSs III are homodimeric proteins with two independent active sites which catalyze a series of decarboxylation, condensation and cyclization reactions to generate polyketides of different lengths. The PKSs III utilize two substrates: a CoA-linked starter unit (usually aromatic CoA) and an acetyl unit (derived from malonyl-CoA) to form an intermediate linear product. Once formed, the linear polyketide is cyclized in the same active site cavity to form the final product (Jez et al., 2001). In a phylogenetic tree, the PKSs from angiosperms fall into two clusters. One cluster comprises chalcone synthase (CHS) and the other cluster contains the functionally divergent enzymes including biphenyl synthase (BIS) and benzophenone synthase (BPS), but also many different CHS-like proteins. The two groups originate from an ancient duplication of an ancestral gene.



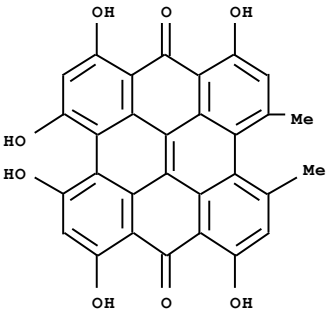
A



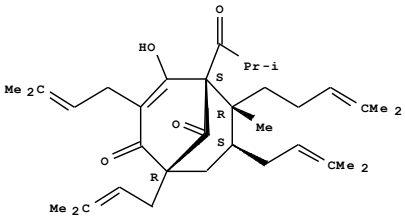
B



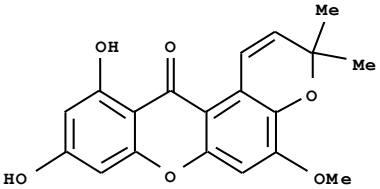
C



D



E



F

Fig. 2: A, chlorogenic acid; B, quercetin; C, quercetrin; D, hypericin; E, hyperforin; F, paxanthone.

1.4.a Type III PKSs and polyketide metabolites in *H. perforatum*

Analyzing the active components of *H.perforatum* extracts, it is possible to detect the activity of at least four different PKSs: CHS, BPS, isobutyrophenone synthase (BUS) and octaketide synthase (OKS).

CHS provides the first committed step in flavonoid biosynthesis by catalyzing the sequential decarboxylative addition of three acetate units from malonyl-CoA to a *p*-coumaroyl-CoA starter molecule derived from phenylalanine *via* the general phenylpropanoid pathway.

BUS stepwise condenses one unit of isobutyril-CoA with three units of malonyl-CoA to give the phlorisobutyrophenone which which is the precursor of hyperforin. OKS is suggested to condensate one acetyl-CoA with seven malonyl-CoA to form an octaketide chain that subsequently undergoes cyclizations and decarboxylation to form emodin anthrone, precursor of the hypericin. BPS catalyzed the condensation of one unit of benzoyl-CoA with three molecules of malonyl-CoA to form a linear tetraketide intermediate which undergoes Claisen condensation to form the phlorbenzophenone (Liu et al., 2003). Phlorbenzophenone is the precursor of many important bioactive compounds. When it undergoes regioselective ring closures by a cytochrome P450, it is converted in xanthones.

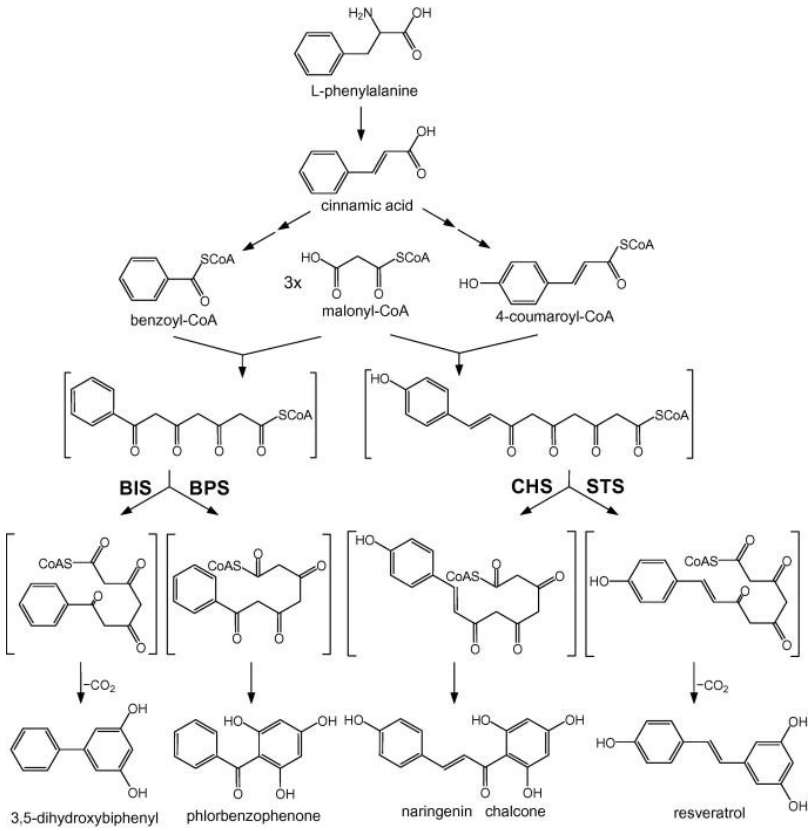


Fig.3 Type III Polyketide Synthases catalyzing the formation of very important secondary metabolites. BIS, Biphenyl Synthase; BPS, Benzophenone Synthase; CHS, Chalcone Synthase; STS, Stilbene Synthase.

1.5 Xanthones

Xanthones are heterocyclic compounds having a dibenzo-γ-pyrone skeleton as basic structure. The name xanthones designates a group of secondary metabolites normally found in a restricted assembly of higher plants (mainly belonging to the families of Hypericaceae and Gentianaceae) fungi and lichens.

1.5.a Xanthone biosynthesis

The biosynthesis of xanthones was studied for the first time in cell cultures of *Centaurium erythraea* and then in cell cultures of *Hypericum androsaemum* L. These two systems showed two alternative pathways for xanthone biosynthesis. BPS from *C. erythraea* catalyses the condensation of one molecule of 3-hydroxybenzoyl-CoA with three molecules of malonyl-CoA to form the 2,3',4,6-tetrahydroxybenzophenone, which is then converted in 1,3,5-trihydroxyxanthone by a xanthone synthase (Beerhues, 1996).

BPS from *H. androsaemum* prefers benzoyl-CoA as starter substrate and catalyses the stepwise condensation of three molecules of malonyl-CoA with one molecule of benzoyl-CoA to form the 2,4,6-trihydroxybenzophenone, which is then converted in 2,3',4,6-tetrahydroxybenzophenone by a benzophenone 3'-hydroxylase. The 2,3',4,6-tetrahydroxybenzophenone is then converted in a variety in a variety of important prenylated plant metabolites (Fig.4) and in 1,3,7-trihydroxyxanthone (Schmidt et al., 1997).

Both 1,3,5- and 1,3,7-trihydroxyxanthones are precursors of higher plant xanthones, thus the 2,3',4,6-tetrahydroxybenzophenone represents a crucial point for xanthone biosynthesis.

Analyzing the two alternative pathways described above, it emerges that the conversion of benzophenone in xanthones is dependent on the 3-hydroxy group. That's why, when benzoylCoA acts as substrate, 3-hydroxylation occurs at the benzophenone level (Beerhues, 1996).

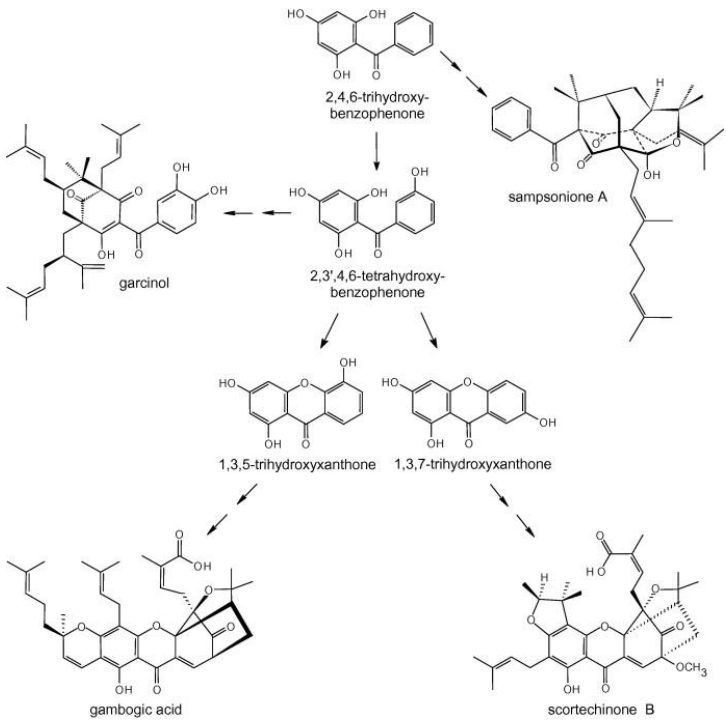


Fig.4 2,3',4,6-tetrahydroxybenzophenone is converted in a variety of important metabolites and in 1,3,5- and 1,3,7-trihydroxyxanthone, the precursors of all the other xanthones.

1.5.b Biological activities of xanthones

One of the undisputed function of the phenolic compounds is their role in protecting plants against microbial invasions. That's why it is possible

to find them as constitutive compounds or as inducible defense in response to microbial attack (phytoalexins). Xanthones have been detected as constitutive compounds, but studies investigating their inducibility as phytoalexins *in vivo* are missing, while many *in vitro* studies suggest their role in the inducible response to microbial attacks. Cell cultures represent an important system to investigate the regulation of secondary metabolite biosynthetic pathways. When treated with pathogens or with pathogen derived substances, it is possible to induce a defense response in plant cells and detect the activation or inhibition of biosynthetic pathways. Regarding xanthones, Beerhues and Berger (1995) observed an increase in xanthone accumulation in cell cultures of *Centaurium erythraea* upon elicitation with yeast extract and methyl jasmonate (Meja). Conceicao et al. (2006) and Tocci et al. (2010) reported the increase in xanthone production from *H. perforatum* cell cultures after *Colletotrichum gloeosporioides* cell wall extracts, MeJa, salicylic acid and chitosan elicitation. Franklin et al. (2009) studying xanthone production in response to *Agrobaterium tumefaciens* elicitation in *H. perforatum* cell cultures suggested a double role of xanthones in the defense response as antioxidant and antimicrobial compounds (Fig.5). After the inoculation of *A. tumefaciens* in the cell culture a rapid upregulation of the gene encoding for BPS and the *de novo* production of xanthones were observed. The authors measured the antioxidant and antimicrobial activity of the extracts from elicited cells and they suggested that *in vivo* xanthones on one hand constitute a powerful antioxidant system of protecting the host cells from self-generated

reactive oxigen species (ROS) and on the other hand have also the potential to act as phytoalexins against the pathogen.

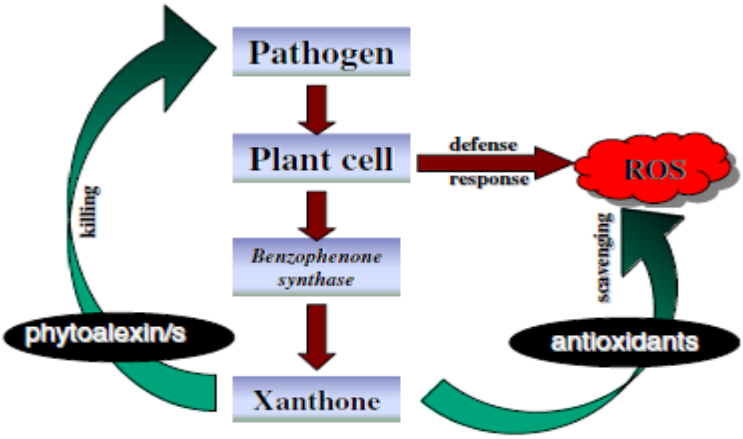


Fig. 5 Proposed role of xanthones in plant defense (Franklin et al., 2009).

1.5.c Pharmacological activities of xanthones

Their role in the plants explains the increased interest in the chemistry of xanthones for the prospect to treat human diseases caused by pathogenic microorganism.

Among the documented pharmacological and biological activities of xanthones:

- **Antifungal, antibacterial, antiviral activities**: prenylated xanthones have been documented the most for their antimicrobial activity. In order to understand their mechanism of action, some prenylated xanthones have been tested against *Bacillus subtilis*, *Staphilococcus aureus* and *Micrococcus luteus*. Data

collected during these studies suggest that isoprenoid substituted xanthones may exert antimicrobial action by reducing the fluidity of outer and inner layers of bacterial cell membranes (Toshio et al., 2004). Not only prenylated xanthones are reported as antimicrobial agents but also some simple oxygenated xanthones and some xantholignoids (Fotie and Bohle, 2006).

With respect to antifungal profile of tested xanthones, it seems to be dependent on the hydroxyl group on ring A and B. This finding is supported by studies carried out testing mangostin against plant pathogenic fungi *Fusarium oxysporum vasinfectum*, *Alternaria tenuis* and *Dreschlera oryzae*. The alkylation of C-3 and C-6 hydroxyl groups resulted in the considerably reduction of the antifungal activity, and replacement with alkyl groups of increasing chain length correlates with decreasing inhibitory activity (Gopalakrishnan et al.1997) Prenylated xanthones are not the only xanthones reported for their antimicrobial activity. Some simple oxygenated xanthones and some xantholignoids have proved to be great antimicrobial agents (Fotie and Bohle, 2006).

- **Antimalarial activity**: hydroxyxanthones have been identified as novel antimalarial agents. The malaria parasite has a limited capacity for de novo synthesis of aminoacids and its survival is dependent on hemoglobin proteolysis. The products of hemoglobin proteolysis are amino acids and heme. The amino acids are incorporated by the parasite and heme oxides to hematin. Free hematin can damage the cellular metabolism inhibiting enzymes involved in the surveillance of cell integrity.

Plasmodial species lack the heme oxygenase involved in the heme catabolism. To protect from heme, *Plasmodium* sequesters the product into a inner crystal (hemozoin). Hydroxyxanthones are believed to exert their activity by complexation of the heme group and inhibition of hemozoin crystal formation. This process is able to induce the death of the parasite as a consequence of the toxicity of the free hematin (Fotie and Bohle, 2006).

- **Anti-inflammatory activity**: some hydroxyxanthones have been shown to exert anti-inflammatory activity by inhibiting the expression of cell adhesion molecules (CAM), which are responsible of the recruitment of leukocytes to the site of inflammation (Madan et al., 2004).
- **Antioxidant activity**: many phenolic compounds, including xanthones, have been shown to act as scavengers of various oxidizing species. Among xanthones, prenylated xanthones are the most widely reported for their antioxidant activity (Fotie and Bohle, 2006).
- **Enzyme inhibitory activity**: different xanthones have been found to be able to inhibit the action of very important enzymes involved in human pathologies. A very important activity is inhibition of monoaminooxidase (MAO). 1,6-dihydroxyxanthone and 1,3,6-trihydroxyxanthone exhibited potent inhibitory activity toward MAO, an enzyme which plays an important role in the regulation of some neurologically active

amines (Ohishi et al, 2000). The inhibitors of MAO are useful in the therapy of psychosis, depression and schizophrenia.

- **Vascular activity**: xanthones and xanthone derivatives have been shown to have beneficial effects on some cardiovascular diseases like ischemic heart disease, atherosclerosis, hypertension and thrombosis (Jiang et al., 2004). Their beneficial effects are due to the antioxidant, anti-inflammatory, anti-platelet and antithrombic activities.

1.5.d Xanthones in *H. perforatum*

Table 1 shows the list of xanthones reported for the species *H. perforatum*. Chemical analysis revealed that xanthones are accumulated mostly in the roots, but studies to individualize cells, tissues or organs responsible of their synthesis are missing. The investigation of biological properties of xanthones from *H. perforatum* revealed an interesting antifungal activity against plant pathogenic fungi belonging to the genera *Phomopsis* and *Colletotrichum* (Crockett el a., 2010). Unfortunately xanthones are present in *H. perforatum* extracts only in traces, not exploitable for applicative uses.

Tab. 1 Xanthones in *H. perforatum* plants

Xanthones	**Aerial parts**	**Roots**	**References**
Mangiferin	X		Kitanov & Nedialkov 1998
Kielcorin		X	Nielsen & Arends 1978; Crockett et al 2010, Tocci et al., Unpublished
1,3,6,7-tetrahydroxyxanthone		X	Sparenberg et al., 1993; Tocci et al., Unpublished
1,3,5-trihydroxyxanthone		X	Murunganandam et al., 2000
1-hydroxy-3,5-dimetoxyxanthone		X	Murunganandam et al., 2000
1,3,5-trihydroxyxanthone		X	Murunganandam et al., 2000; Tocci et al., Unpublished
Paxanthone		X	Tocci et al. Unpublished
1,3,5,6-tetrahydroxyxanthone		X	Tocci et al. Unpublished
5-O-methyl-2-deprenylrheediaxanthone B		X	Crockett et al 2010; Tocci et al. Unpublished
1,7-dihydroxyxanthone		X	Tocci et al. Unpublished
4,6-dihydroxy-2,3-dimetoxyxanthone		X	Crockett et al 2010

1.6 Biotechnology advances for the production of xanthones in *Hypericum perforatum*

The plant kingdom has historically been a major source of bioactive compounds for medicine, food additives, pigments, insecticides, cosmetics, and fine chemicals. However, the recovery of bioactive compounds from natural sources is often problematic, in that the extract may contain only very small quantities or its composition may vary with the season or the environment. To obtain qualitatively and quantitatively standardized extracts, plant biotechnology can represent a valuable alternative. The advantages of this technology over the conventional agricultural production are as follows:

- It is independent of geographical and seasonal variations and various environmental factors.
- It offers a defined production system, which ensures the continuous supply of products, uniform quality and yield.
- It is possible to produce novel compounds that are not normally found in parent plant.
- Efficient downstream recovery and product.
- Rapidity of production.
- Plant cell can perform stereo- and regiospecific biotransformations for the production of novel compounds from cheap precursors.

For these reasons in the decades many studies have been focalized on plant cell cultures as a possible method to produce plant secondary metabolites of commercial interest (Verpoorte et al., 1993, 1998;

Buitelaar and Tramper,1992; Lipsky, 1992; Su, 1995). However, for many of the desired compounds, the production from cell cultures is too low. This is usually because some compounds require tissue differentiation to be correctly synthesized.

Many authors demonstrated that in undifferentiated calli and suspended cells of *H. perforatum* xanthones are the main metabolic products accumulated (Dias et al., 1999; Dias et al., 2001; Pasqua et al., 2003; Conceicao et al., 2006). Cell cultures of *H. perforatum* have been shown to synthesize mainly xanthones characterized by a 1,3,5,6- and/or a 1,3,6,7-oxygenation pattern and that their production is highly influenced by hormone supplementation (Dias et al., 2001). In particular Dias et al. (2001) and Tocci et al. (2010) observed a positive linear relationship between naphthalene acetic acid concentration in the culture medium and xanthone accumulation. Moreover Mulinacci et al. (2008) showed that also for *H. perforatum* subsp. *angustifolium* xanthones are the main metabolites produced in calli and suspended cells. However all of the studies mentioned above reported the production of only small amount of xanthones not exploitable for applicative purposes.

The elicitation is a technique commonly used in plant biotechnology to enhance secondary metabolite production and it has been applied to suspended cell cultures of *H. perforatum.* Conceicao et al. (2006) found a substantial increase in xanthone production after treatment of *H. perforatum* cell cultures both with methyl jasmonate and *Colletotrichum gloeosporioides*, which is a natural fungal pathogen of the species. The same founding was observed by Franklin et al. (2008) and Tocci et al. (2010) when using respectively *A. tumefaciens* and chitosan as elicitors. However

even if elicitation has represented a step forward for the enhancement of xanthone production from cell cultures of *H. perforatum*, their amount was still far away from what is necessary for applicative uses.

For the production of medicinally important compounds, organ cultures represents a valid alternative being relatively more stable (Roja, 1994). By transformation with *Agrobacterium rhizogenes*, the so called "hairy roots", can be obtained. Hairy roots can grow without plant growth hormones, and are good producers of the typical root secondary metabolites, but their induction requires the use of selectable markers, often resistence genes, and this has raised question for human health. With respect of this point of view, the adventitious root cultures are a promising reliable way for production of pharmaceutically and nutraceutically important metabolites. Moreover many efforts have been made to induce hairy roots production from *H. perforatum* explants (Vinterhaltern et al., 2006; Franklin et al., 2008), but the poor results obtained suggest that the species is recalcitrant to the transformation with *A. rhizogenes*.

1.6.a Adventitious root cultures

Adventitious roots have been successfully induced in many plant species (Table 2) and cultured for the production of high value secondary metabolites of pharmaceutical, nutraceutical and industrial importance. Although root cultures of *H. perforatum* have also been established by other authors (Goel et al. 2009; Cui et al. 2010; Tocci et al., 2011), the capacity of these cultures to produce xanthones has never been explored. The production of secondary metabolites from adventitious roots involves four stages (Fig.6). The first stage is the induction of adventitious roots from plant explants. The induction can be direct from

many type of explants (roots, rhizome, leaves, stem) or indirect involving callus formation and re-differentiation in roots. Both direct and indirect rhizogenesis are achieved by exposure of plant tissues to various concentrations of auxins like indol-3-acetic acid, indole-3-butyric acid, naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid, which are able to induce both growth of pre-existing roots (e.g. stimulating branching) or adventitious root formation.

When roots are formed, the second stage is the cultivation in liquid medium in flasks or small bioreactors. This phase includes the optimization of growth kinetics of adventitious roots and the evaluation of secondary metabolite accumulation. The third stage is the development of strategies to enhance metabolite production. The secondary metabolism can be stimulated by elicitors, which are signals triggering the activation of biosynthetic pathways for secondary metabolites. The use of biotic and abiotic elicitors reduce the process time to attain high product concentrations (Ramachandra et al 2002). The stress signaling molecules like methyl jasmonate or salicylic acid, or molecules of fungal, bacterial and yeast origins (chitosan, glycoproteins, polysaccharides) and salts of heavy metals are frequently used with success in elicitation experiments. Once biomass and secondary metabolism parameters have been optimized, the fourth stage is the scale-up process of the cultures in a bioreactor.

Regarding adventitious roots, due to their structural features and metabolite localization characteristics, they need different type of reactors than the ones used for plant cell cultures. The main problem in bioreactor is the tendency of roots to form clumps. Moreover it is

impossible to define one best bioreactor type because the rheological properties vary from one species to another.

Tab.2 Cultures of adventitious roots for the production of important metabolites

Plant species	**Metabolites**	**Importance**	**References**
Anthemis nobilis	Geranyl isovalerate	Essential oil, fragrance, anti-inflammatory	Omoto et al., 1998
Cornus capitata	Tannins	Antioxidant	Tanaka et al., 2001
Duboisia myoporoides, D. leichhardtii hybrid	Scopolamine, hyoscyamine	Spamolytic, kydriatic agents	Yoshimatsu et al., 2004
Echinacea purpurea, E. angustifolia	Caffeic acid derivatives	Immunostimulant, Anti-inflammatory, anti-oxidant	Wu et al., 2006
Iris germanica	Irigenin, Iristectorigenin A (Flavonoids)	Anti-inflammatory, anti-cancer	Akashi et al., 2005
Scopolia parviflora	Hyoacyamine (Alkaloid)	Anticholinergic	Kang et al. 2004, Min et al., 2007
Panax ginseng	Ginsenosides (Saponins)	Immunostimulant, Anti-inflammatory,anti-oxidant, anti-cancer, anti-fatigue	Choi et al., 2000; Kim 2002; Kim et al., 2005; Jeong et al., 2006; Son et al., 1999
Panax notoginseng	Saponins	Immuntostimulant, anti-cancer	Gao et al., 2005
Raphanus staivsus	Anthocyanin	Food coloring	Betsui et al., 2004
Rhus javanica	Galloylglucoses, riccionidin A	Anti-oxidants	Taniguchi et al., 2000

The choice of the bioreactor has to be done independently for each species considering all the parameters that could affect the productivity of the system. The downstream process for the recovery of metabolites represents the final step of the production system. The process should be as simple as possible. The metabolites of interest can be released in the culture medium or accumulated by the organ. The first case is the most desirable and convenient for the recovery of metabolites and for the conservation of root biomass, allowing a continues culture without loss of plant material. At the end of culture period, in fact, the liquid medium is collected and processed for the extraction of metabolites, while the biomass is ready for a new productive cycle. In the second case, the release of metabolites can be stimulated by wide variety of permeabilizing agents, such as organic solvents (isopropanol, dimethylsulfoxyde) or by the help of other permeabilization methods, including ultrasonication, electroporation and ionophoretic release. When these tools don't work, it is necessary to collect and process the biomass, resulting in loss of part of it. At the end of a culture period, part of the biomass is transferred to new medium to start a new productive cycle, while part is destroyed for metabolite recovery. All the stages described above represent a bottle-neck to be considered in the evaluation of cost/benefit advantages for production of secondary metabolites from root cultures at industrial levels. (Murthy et al., 2008).

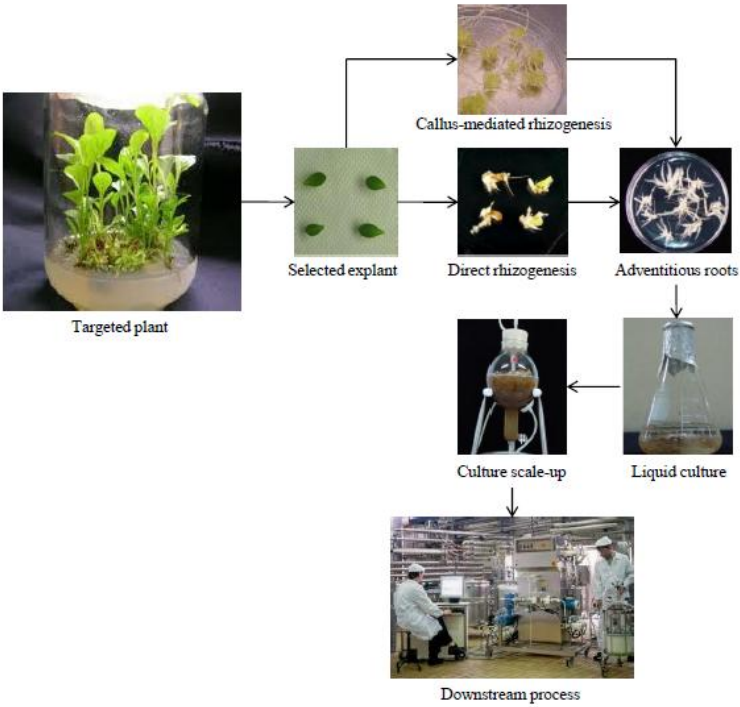


Fig.6 Steps involved in the development and establishment of adventitious root cultures.

Bibliography

References

- Akashi T, Ishizaki M, Aoki T, Ayabe S. Isoflavonoidproduction by adventitious root cultures of *Iris germanica* (Iridaceae). *Plant Biotechnol*, 2005, 22: 207−215.
- Baroni Fornasiero R, Bianchi A, Pinetti A. Anatomical and ultrastuctural observations in *Hypericum perforatum* L. leaves. *J Herbes, spices med plants*, 1998, 5: 21-33.
- Beerhues L, Berger U. Differential accumulation of xanthones in methyl-jasmonate- and yeast-extract-treated cell cultures of *Centaurium erythraea* and *Centaurium littorale*. *Planta*, 1995, 608-612.
- Beerhues L. Benzophenone synthase from cultured cells of *Centaurium erythraea*. *FEBBS Lett*, 1996, 383: 264-266.
- Betsui F, Tanaka-Nishikawa N, Shimmomura K. Anthocyanin production in adventitious root cultures of *Raphanus sativus* L. cv. Peking Koushin. *Plant Biotechnol*, 2004, 21: 387−391.
- Bombardelli E, Morazzoni P. *Hypericum perforatum*. *Fitoterapia,* 1995, 66: 43-68.
- Buitelaar RM, Tramper J. Strategies to improve the production of secondary metabolites with plant cell cultures : a literature review. *J Biotech*, 1992, 23:111-141.
- Chatterjee SS, Bhattacharya SK, Wonnemann M. Hyperforin as a possible antidepressant component of hypericum extracts. *Life Sciences*, 1998, 63: 499-510.

- Choi SM, Son SH, Yun SR, Kown OW, Seon JH, Paek KY. Pilot scale culture of adventitious roots of ginseng in abioreactor system. *Plant Cell Tissue Organ Cult*, 2000, 62:187−193.
- Conceicao LFR, Ferreres F, Tavares RM, Dias ACP. Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloesporioides* elicitation. *Phytochem*, 2006, 67: 149-155.
- Crockett S, Robson NK. Taxonomy and Chemotaxonomy of the Genus *Hypericum*. *Med Aromat Plant Sci Biotechnol*, 2011, 5: 1–13.
- Crockett SL, Poller B, Tabanca N, Pferschy-Wenzig EM, Kunert O, Wedge DE, Bucar F. Bioactive xanthones from the roots of *Hypericum perforatum* (common St John's wort). *J Sci Food Agric*, 2011, 91:428-434.
- Cui XH, Chakrabarty D, Lee AJ, Paek KY. Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. *Bioresour Techonol*, 2010, 101:4708-4716.
- Fotie J, Bohle DS. Pharmacological and biological activities of xanthones. *Antinfective Agents Med Chem*, 2006, 5: 15-31.
- Franklin G, Conceição LFR, Kombrink E, Dias ACP. *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta*, 2008, 1401-1408.
- Franklin G, Conceição LF, Kombrink E, Dias AC. Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant

and antimicrobial protection upon biotic stress. *Phytochem*, 2009, 70: 60-68.

- Gao X, Zhu C, Jia W, Gao W, Qiu M, Zhang Y, Xiao P. Induction and characterization of adventitious roots directly from the explants of *Panax notoginseng*. *Biotechnol Lett*, 2005, 27:1771−1775.
- Goel MK, Kulreja AK, Bishut NS. *In vitro* manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. *Plant Cell Tiss Organ Cult*, 2009, 96: 1-9.
- Gopalakrishnan et al.1997 in: Fotie J, Bohle DS. Pharmacological and biological activities of xanthones. *Antinfective Agents Med Chem*, 2006, 5: 15-31.
- Jang et al., 2004 in Fotie J, Bohle DS. Pharmacological and biological activities of xanthones. *Antinfective Agents Med Chem*, 2006, 5: 15-31.
- Jeong CS, Chakrabarty D, Hahn EJ, Lee HL, Paek KY. Effects of oxygen, carbon dioxide and ethylene on growthand bioactive compound production in bioreactor culture of ginseng adventitious roots. *Biochm Eng J*, 2006, 27:252−263.
- Jez JM, Ferrer JL, Bowman ME, Austin MB, Schröder J, Dixon RA, Noel JP. Structure and mechanism of chalcone synthase-like polyketide synthases. *J Ind Microbiol Biotech*, 2001, 27 393-398.

- Kang SM, Jung HY, Kang YM, Yun DJ, Bahk JD, Yang JK,Choi MS. Effects of methyl jasmonate and salicylic acid onthe production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia praviflora. Plant Sci*, 2004, 166: 745−751.
- Kim YS, Hahn EJ, Murthy HN, Paek KY. Adventitious rootgrowth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. *Biotechnol Lett*,2005, 26: 1619−1622.
- Kim YS, Yeung EC, Hahn EJ, Paek KY. Combined effects of phytohormone, indole-3-butyric acid, and methyl jasmonateon root growth and ginsenoside production in adventitiousroot cultures of *Panax ginseng* C.A. Meyer. *Biotechnol Lett*, 2007, 29: 1789−1792.
- Kim YS. Production of ginsenosides through bioreactorcultures of adventitious roots in ginseng (*Panax ginseng* C. A.Meyer). Ph. D. thesis, Chungbuk National University,Chenogju, South Korea, 2002, 1−137.
- Kitanov GM, Nedialkov PT. Mangiferin and isomangiferin in some *Hypericum* species. *Biochem Syst Ecol*, 1998, 26: 647-653.
- Lavie G, Mazur Y, D Lavie, D Meruelo. The chemical and biological properties of hypericin a compound with a broad spectrum of biological activities. *Med Res Rev*, 1995, 15: 111-119.
- Lipsky AK. Problems of optimisation of plant cell culture processes. *J Biotech*, 1992, 26: 83-97.

- Liu B, Falkenstein-Paul H, Schmidt W, Beerhues L. Benzophenone synthase and chalcone synthase from *Hypericum androsaemum* cell cultures: cDNA cloning, functional expression, and site-directed mutagenesis of two polyketide synthases. *Plant J*, 2003, 34: 847-855.
- Madan B, Singh I, Kumar A, Prasad AK, Raj HG, Parmar VS, Ghosh B. Xanthones as inhibitors of microsomal lipid peroxidation and TNF-alpha induced ICAM-1 expression on human umbilical vein endothelial cells (HUVECs). *Bioorg Med Chem*, 2002, 10: 3431-3436.
- Males Z, Brantner A, Sovic K, Pilepic K, Plazibat M. Usporedna fitokemijska i antimikrobna istraživanja širokolisne i uskolisne rupičaste pljuskavice [*Hypericum perforatum* L. subsp. *perforatum* et H. *perforatum* subsp. *angustifolium* (DC.) Gaudin]. *Acta Pharmaceutica*, 2006, 56, 359-367.
- Mathis C, Ourrison G. Etude chimio-taxanomique du genre *Hypericum* I. Rèpartition de Hypèricine. *Phytochemistry,* 1963, 2: 157-171.
- McCutcheon AR. An Exploration of Current Issues in Botanical Quality: A Discussion Paper. Natural Health Products Directorate, Health Canada, 2002.
- Melzer R, Fricke U, Hoelzl J. Vasoactive properties of protocyanidins arteries. *Arznm.-Forsch./Drug Res*, 1991, 41: 481-483.

- Min JY, Jung HY, Kang SM, Kim YD, Kang YM, Park DJ,Prasad DT, Choi MS. Production of tropane alkaloids bysmall-scale bubble column bioreactor cultures of *Scopolia parviflo*ra adventitious roots. *Bioresource Technol*, 2007, 98:1748−1753.
- Murthy HN, Hahn EJ, Paek KY. Adventitious Roots and Secondary Metabolism. *Chin J Biotech*, 2008, 24: 711-716.
- Muruganandam A, Ghosal S, Batthacharya S. The role of xanthone in the antidepressant activity of *Hypericum perforatum* involving dopaminergi and serotonergic systems. *Biogenic amines* 2000, 15: 553-567.
- Nielsen H, Arends P**.** Structure of the xanthone lignoid kielcorin. *Phytochem*, 1978, 17: 2040-2041.
- Ohishi N, Suzuki T, Ogasawara T, Yagi K. Xanthone derivatives as inhibitors for monoamine oxidase. *J Mol Cat*, 2000, 10: 291-294.
- Omato T, Asai I, Ishimaru K, Shimomura K. Geranylisovalerate accumulation in adventitious root cultures of *Anthemis nobilis*. *Phytochem*, 1998, 48: 971−974
- Pignatti S. La Flora d'Italia. Edagricoltore, 1982.
- Ramachandra Rao S, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. *Biotech Adv*, 2002, 20: 105-153.

- Roja G, Heble MR. The quinoline alkaloids camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of *Nothapodytes foetida*. *Phytochem*, 1994, 36: 65-66.
- Schmidt W, Beerhues L. Alternative pathways of xanthone biosynthesis in cell cultures of *Hypericum androsaemum* L. *FEBBS Lett*, 1997, 420: 143-146.
- Soelberg J, Jørgensen LB, Jäger AK. Hyperforin Accumulates in the Translucent Glands of *Hypericum perforatum*. *Ann Bot*, 2007, 99: 1097-1100.
- Son SH, Choi SM, Hyung SJ, Yun SR, Choi MS, Shin EM, Hong YP. Induction and cultures of mountain ginsengadventitious roots and AFLP analysis for identifying mountain ginseng. *Biotechnol Bioprocess Eng*, 1999, 4:118−123.
- Sparenberg B. PhD thesis 1993, University of Marburg, Germany.
- Stevens PF. Clusiaceae-Guttiferae in "*The Families and Genera of Vascular Plants*", 2007, 9: 48-66. Springer Editor.
- Su W. Bioprocessing technology for plant cell suspension cultures. *App Biochem Biotech*, 1995, 50:189-203.
- Tanaka N, Tanaka T, Fujioka T, Fujii H, Mishashi K,Shimomura K, Ishimaru K. An ellagic compound andirridoids from *Cornus capitata* root cultures. *Phytochem*, 2001, 57: 1287−1291.
- Taniguchi S, Yazaki K, Yabu-uchi R, Kawaskami K, Ito H,Hatano T, Yoshida T. Galloylglucoses and reccionidin A

in *Rhus javanica* adventitious root cultures. *Phytochem*, 2000, 53: 357−363.

- Tocci N, Ferrari F, Santamaria AR, Valletta A, Rovardi I, Pasqua G. Chitosan enhances xanthone production in *Hypericum perforatum* subsp. *angustifolium* cell cultures. *Nat Prod Res*, 2010, 24: 286-293.
- Tocci N, Simonetti G, D'Auria FD, Panella S, Palamara AT, Valletta A, Pasqua G. Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity. *Appl Microbiol Biotechnol*, 2011, 91: 977-987.
- Toshio et al., 2004 in: Fotie J, Bohle DS. Pharmacological and biological activities of xanthones. *Antinfective Agents Med Chem*, 2006, 5: 15-31.
- Verpoorte R, van der Heijden R, Schripsema J, Hoge JHC, Ten Hoopen HJG. Plant Cell Biotechnology for the Production of Alkaloids: Present Status and Prospects. *J Nat Prod*, 1993, 56: 186-206.
- Verpoorte R. Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. *Drug Discovery Today*, 1998, 3: 232-238.
- Vinterhalter B, Ninković S, Cingel A, Vinterhalter D. Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS. *Biol Plan*, 2006, 50:767-770.

- Wu CH, Dewir YH, Hahn EJ, Paek KY. Optimization of culturing conditions for the production of biomass andphenolics from adventitious roots of *Echinacea angustifolia*. *J Plant Biol*, 2006, 49: 193−199.
- Wu CH, Murthy HN, Hahn EJ, Paek KY. Enhancedproduction of caftaric acid, chlorogenic acid and cichoricacid in suspension cultures of *Echinacea purpurea* by themanipulation of incubation temperature and photoperiod. *Biochem Eng J*, 2007b, 301−303.
- Wu CH, Murthy HN, Hahn EJ, Paek KY. Large-scalecultivation of adventitious roots of *Echinacea purpurea* in air-lift bioreactors for the production of chichoric acid, chlorogenic acid and caftaric acid. *Biotechnol Lett*, 2007a,29:1179−1182.
- Yoshimatsu K, Sudo H, Kamada H, Kiuchi F, Kikuchi Y,Sawada J, Shimomura K. Tropane alkaloid production andshoot regeneration in hairy and adventitious root cultures of Duboisia myoporoides – D. leichhardtii *Hybrid. Biol PharmBull*, 2004, 27: 1261−1265.

Chapter 2:

Aims of the project

Aims of the research project

- Xanthones are secondary metabolites showing many biological activities. *Hypericum perforatum* is a medicinal plant intensively studied for its curative properties. In spite of this, little is known about xanthone biosyntheisis in the species. The aim of the present research project has been to investigate xanthone biosynthesis *in planta* and in *in vitro* systems of *Hypericum perforatum* subsp. *angustifolium* at chemical and molecular levels. Moreover, organ and tissue localization, through whole mount *in situ* hybridization, of the gene transcript of benzophenone synthase, key enzyme of xanthone biosynthesis, have been carried out both in plantlets and in *in vitro* roots.

In parallel, a biotechnological system for xanthone production at exploitable levels from root cultures has been established and optimized using different elicitors such as chitosan and derivatives. Root extracts and isolated xanthones have been tested in antifungal therapy against human pathogenic fungi. The objectives have been pursued as follows:

1. **Xanthone biosynthesis and accumulation in tissues and organs.**

The study has been carried out through:

- To individuate xanthone accumulation organs chemical analysis were carried out both in plants grown-wild and grown under controlled conditions;
- To individuate tissues and organs responsible for xanthone biosynthesis, cloning, functional charcaterization and localization of

benzophenone synthase (key enzyme of xanthone biosynthesis) gene transcript, through whole mount *in situ* hybridization, have been carried out both in plantlets and *in vitro* roots.

2. **Establishment of root cultures for xanthone production at exploitable levels for applicative purposes.**

The study has been carried out through:

- Establishment of root cultures;
- Evaluation of regenerated roots capability to produce xanthones;
- Optimization of culture conditions to increase biomass and xanthone yields;
- Optimization of xanthone production by elicitation with chitosan and its derivatives.

3. **Evaluation of antifungal activity of xanthone-rich extracts against human pathogenic fungi.**

The tests have been carried out through:

- Determination of the Minimum Inhibition Concentration (MIC) at which the xanthone-rich extracts are able to inhibit the growth of human fungal pathogens (*Candida* spp., dermatophytes and *Cryptococcus neoformans*).

Chapter 3:

Chemical composition and antifungal activity of *Hypericum perforatum* subsp *angustifolium* roots from plants collected in field and grown under controlled conditions

Manuscript submitted to Plant Biosystems (Official Journal of the Società Botanica Italiana)

Chemical composition and antifungal activity of *Hypericum perforatum* subsp *angustifolium* roots from plants grown wild and under controlled conditions

Tocci Noemi[1*], Simonetti Giovanna[2*], D'Auria Felicia Diodata[2], Panella Simona[2], Palamara Anna Teresa[2], Ferrari Franco[1], Pasqua Gabriella[1]

[1]Department of Environmental Biology, [2]Department of Public Health and Infectious Diseases, "Sapienza" University of Rome; Piazzale Aldo Moro 5; 00185 Rome, Italy

[*] The authors Noemi Tocci and Giovanna Simonetti contributed equally to this work.

Abstract

The medicinal properties of the aerial parts of *Hypericum perforatum* subsp *angustifolium* have been extensively investigated, yet little is known about the chemical composition or potential uses of the root extracts. In the present study, xanthone production in plants grown wild in different areas and in plantlets grown under controlled conditions was investigated. Chemical analyses performed on wild grown plants revealed that xanthones were mainly accumulated in the roots. We detected 1,7-dihydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone and paxanthone (the first report of these xanthones in this species), together with 5-O-methyl-2deprenylrheediaxanthone B, kielcorin and 1,3,6,7-tetrahydroxyxanthone. Wild grown plants showed variability and a low yield in xanthone content in the roots. Compared to the roots of wild grown plants, the roots of plantlets grown under controlled conditions

had a 27-times greater xanthone accumulation. Moreover, they produced toxyloxanthone B, which was not detected in the roots from wild plants; kielcorin was not detected. Since xanthones are known for their antifungal activity, the extracts from both samples were tested against the human fungal pathogens as *Candida albicans*, *Candida* spp., dermatophytes and *Cryptococcus neoformans*. The root extracts from plantlets grown under controlled conditions showed greater antifungal activity. In conclusion, the roots of plants grown under controlled conditions have greater antifungal activity, especially against *Candida* species, and this activity is positively correlated with xanthone accumulation.

Key words: *Hypericum*, roots, *Candida,* dermatophytes, *Cryptococcus,* antifungal.

Introduction

Hypericum perforatum L. (Hypericaceae) is a medicinal plant which is distributed throughout Eurasia (Cervo et al., 2002). The medicinal properties of the aerial parts of *H. perforatum* have been extensively investigated, and several classes of secondary metabolites have been identified, including naphthodianthrones (i.e., hypericin and pseudohypericin), flavonol glycosides (i.e., isoquercitrin and hyperosid), biflavonoids (i.e., amentoflavone), and phloroglucinol derivatives (i.e., hyperforin and adhyperforin) traces of xanthones have also been found (Nahrstedt and Butterweck 2010). The interest in *H. perforatum* has been mainly focused on its antidepressant effects. Although the medicinal properties of the aerial parts have been extensively investigated, little is

known about the chemical composition or the potential applications of the root extracts. Recently, Crockett et al., (2011) isolated three xanthones from root extracts of *H. perforatum*: 1,6-dihydroxy-5-methoxy-4',5'-dihydro-4',4',5'-trimethylfurano-(2',3':3,4)-xanthone; 4,6-dihydroxy-2,3-dimethoxyxanthone; and *cis*-kielcorin; and they evaluated their antifungal activity against plant pathogenic fungi. Tocci et al. (2011 and 2012) demonstrated that extracts from *in vitro* regenerated roots of *H. perforatum* subsp. *angustifolium*, treated with chitosan, showed antifungal activity against human fungal pathogens, in particular, *Candida* species, *Cryptococcus neoformans* and dermatophytes. They also found a positive correlation between xanthone accumulation and antifungal activity (Tocci et al., 2012). The importance of these results lies in the fact that, in the past 30 years, there has been a dramatic increase in the incidence of fungal infections (Pfaller et al., 2010) and in the number of pathogenic fungi that are resistant to commercial antifungal compounds; consequently, there has been a greater need for effective therapy (De Vita et al., 2012), which has led to the search for therapeutic alternatives among natural products (Oliveira et al., 2012).

The objective of the present study was to evaluate the chemical composition and antifungal activity of root extracts obtained from *H. perforatum* subsp. *angustifolium* plants, and to compare these characteristics between plants grown wild at different altitudes and in different soil conditions with plants grown *in vitro* under controlled conditions.

Materials and methods

Plant material

Wild grown plants of *H. perforatum* subsp. *angustifolium* were collected at the vegetative stage in two areas outside of Rome, Italy (i.e., Tivoli Terme and Poli). The plants were collected at two different altitudes [68m and 453m above sea level (asl), respectively, in Tivoli Terme and Poli] and in different types of soil (calcareous in Tivoli Terme and volcanic in Poli). To obtain plants grown under controlled conditions, seeds of *H. perforatum* subsp. *angustifolium* were collected from Tivoli Terme; they were surface sterilized in accordance with Zobayed et al. (2004) and then inoculated in flasks containing 100 ml of half strength Murashige and Skoog (MS) (1962) medium (Duchefa Biochemie, Haarlem, the Netherlands) supplemented with 1.5% (w/v) sucrose. The cultures were grown under a photoperiod of 16/8 h (light/dark) at 26°C (photon lux density, 70 μmol m^{-2} s^{-1}).

Extraction and chemical analysis

The roots and aerial parts of the wild grown plants and the roots of two-month-old *in vitro* plantlets were separated and dried at room temperature for 48h until they reached constant weight. All of the solvents used for extraction were purchased from Carlo Erba Reagents, Italy. Each sample consisted of an aliquot of 4 g (DW), which was ground and subjected to three extractions of 24 h with methanol at room temperature under dark conditions. The obtained extracts were evaporated with a rotary evaporator and dissolved in methanol (HPLC grade) for chemical analysis. The HPLC apparatus consisted of a high pressure pump (Waters 1525) and a dual wavelength UV detector (Dual

λ Detector Waters 2487), equipped with a C_{18} column 4.6x150 mm, pore size 5 μm (Waters). The chromatographic separation was performed through a gradient elution consisting of 2.5 % acetic acid in Milli-Q pure water (solution A) and methanol (solution B), according to the method proposed by Dias et al. (1999) with small modifications. Rutin, isoquercetrin and hyperosid were analysed according to the method (method 2) proposed by Kartig et al. (1996), by isocratic elution with a solution of acetonitrile, water and phosphoric acid (16:83:1). The flow rate was kept at 1 ml/min, and metabolites were detected at 260 nm and 320 nm based on UV spectra of flavonoids and xanthones. The identification of the peaks was carried out by the internal standard method. Standard compounds for xanthones (1,3,6,7-, 1,3,5,6,-tetrahydroxyxanthone; 1,7-dihydroxyxanthone; kielcorin; paxanthone and 5-O-methyl-2-deprenylrheediaxanthone B) and flavonoids (chlorogenic acid, quercetrin, quercetin, luteolin, rutin, and isoquercetrin) were kindly provided by Dr. Franco Ferrari. Hyperosid was purchased from Extrasynthese (Geney, France). The concentration of metabolites in the extracts was calculated using calibration curves realized by injecting standards at different known concentrations. Xanthones were quantified as paxanthone-equivalents. For the chemical analysis, the arithmetic mean and standard deviation (SD) of compound concentration (di che cosa?) were calculated.

Organisms

For the antifungal evaluation, we tested strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), the German Collection of Microorganisms (DSMZ, Braunschweig,

Germany) and the Pharmaceutical Microbiology Culture Collection (PMC, Department of Public Health and Infectious Diseases, "Sapienza" University, Rome, Italy). The strains were: *Candida albicans* (ATCC 10231, ATCC 10261, ATCC 3153, ATCC 24433, ATCC 90028, PMC 1011, PMC 1002, and PMC 1010*),* non-*albicans Candida* species *(C. parapsilosis* ATCC 22019, *C. parapsilosis* DSM 11224*, C. tropicalis* DSM 11953, *C. tropicalis* PMC 0908*, C. tropicalis* PMC 0910, *C. glabrata* PMC 0805, *C. krusei* DSM 6128, and *C. krusei* PMC 0613*), Cryptococcus neoformans* (DSM 11959, PMC 2136, PMC 2123, PMC 2115, PMC 2103, PMC 2107, PMC 2102, and PMC 2111), dermatophytes (*Trichophyton mentagrophytes DSM 4870, T. mentagrophytes PMC 6503, T. mentagrophytes PMC 6509, T. mentagrophytes PMC 6531, T. mentagrophytes PMC 6515, T. mentagrophytes PMC 6552, Microsporum gypseum PMC 7331*, and *M. gypseum PMC 7303*). All of the strains were stored and grown in accordance with the procedures of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008a, b).

Antifungal susceptibility assays

In vitro antifungal susceptibility was evaluated for both the total root extracts from the wild grown plants and the two-month-old *in vitro* plantlets, using the CLSI broth microdilution methods (CLSI, 2008a, b). The antifungal reference fluconazole (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was also tested. The final concentration ranged from 0.0625 to 128 μg mL^{-1} for fluconazole and from 1 to 1024 μg mL^{-1} for total dry root extracts. The extracts were dissolved previously in dimethyl sulfoxide at concentrations 100 times higher than the highest desired test concentration and successively diluted in test medium in accordance with

the procedures of the CLSI (CLSI, 2008c). Microdilution trays containing 100 μL of serial two-fold dilutions of root extracts in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, U.S.A) were inoculated with an organism suspension adjusted to attain a final inoculum concentration of 1.0 x 10^3 - 1.5 x10^3 cells mL^{-1} for yeasts and 0.4x10^4 - 5x10^4 CFU mL^{-1} for dermatophytes. The panels were incubated at 35°C and observed for the presence of growth at 48 h (*Candida* spp.) and 72 h (*C. neoformans* and dermatophytes).

The minimal inhibitory concentration (MIC) was defined as, for yeasts, the lowest concentration that showed ≥50% growth inhibition compared with the growth control and, for dermatophytes, the lowest concentration that showed ≥80% growth inhibition compared with the control. The results are expressed as the geometric mean and range of the MIC.

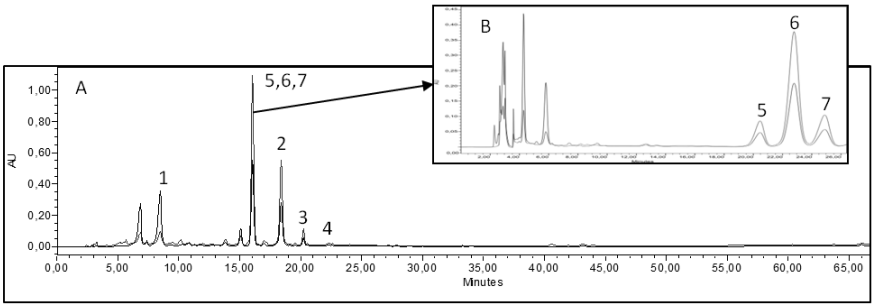


Fig. 1: (A) HPLC chromatogram of the extract of aerial parts of *H. perforatum* grown wild in Tivoli Terme and analyzed using method 1: 1, chlorogenic acid; 2, quercetrin; 3, quercetin; 4, luteolin; (B) HPLC chromatogram of the same extract analyzed using method 2 to resolve the peak at RT 16 min: 5, rutin; 6, hyperoside; and 7, isoquercetrin. The double lines in each chromatogram correspond to the wavelengths 280 nm and 320 nm.

Results

Chemical analysis of the extract of the aerial parts

The chemical analysis of the extracts from the aerial parts of *H. perforatum* subsp. *angustifolium* plants collected in Tivoli Terme (Figure 1) and Poli revealed the presence of phenolic compounds (mainly flavonoids and phenolic acids), however, we found none of the xanthones for which reference compounds were available. The comparison of retention time (RT) and the co-injection with standards led to the identification of chlorogenic acid, quercetrin, quercetin, luteolin, rutin, hyperosid and isoquercetrin. The identified metabolites were quantified according to the compound-specific calibration curves; the concentrations are reported in Table 1 with small differences in the amount of hyperosid (16.562 ± 0.605 mg/g DW in the extract from plants collected in Tivoli Terme and 10.28± 0.65 mg/g DW in the extract from the plants collected in Poli) and quercetin (0.24±0.022 mg/g DW for Tivoli and 0.85± 0.031 mg/g DW for Poli). .

Chemical analysis of the extract of the roots

HPLC analysis revealed that, differently from the aerial parts, the roots of wild plants contained many xanthones (Figure 2), namely, 1,3,6,7-, 1,3,5,6,-tetrahydroxyxanthone; 1,7-dihydroxyxanthone; kielcorin; paxanthone and 5-O-methyl-2-deprenylrheediaxanthone B, together with vanillic acid and luteolin (Table 1). The total amount of xanthones was 2.4 times higher in the sample collected in Tivoli Terme (427.27 ± 26.21 μg/g DW), compared to that collected in Poli (179±20.21 μg/g DW). In the extract from Tivoli Terme, kielcorin and paxanthone were the main xanthones (172.53±11 μg/g DW and 170.74±12.6 μg/g DW,

respectively), whereas the main xanthone in the extract from Poli was 5-O-methyl-2-deprenylrheediaxanthone B (104±12μg/g DW).

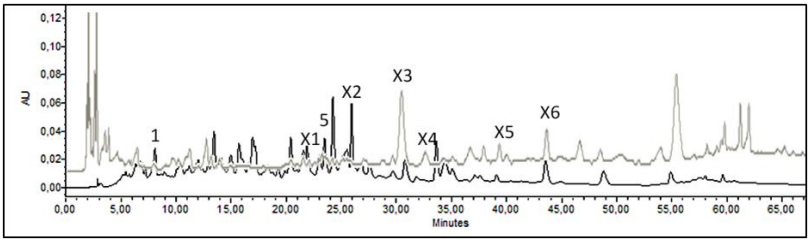


Fig. 2 HPLC chromatogram of root extracts from *H. perforatum* wild grown plants in Tivoli Terme (black) and collected from *in vitro* plantlets (grey). 1, vanillic acid; 5, luteolin; X1, 1,3,4,6-, 1,3,6,7-tetrahydroxyxanthone; X2, kielcorin; X3, 1,7-dihydroxyxanthone; X4, toxyloxanthone B; X5, paxanthone; X6, 5-O-methyl-2-deprenylrheediaxanthone B. The chromatrograms are detected at 280 nm wavelengths (The chromatrograms were performed at a wavelength of 280 nm.

Chemical analysis of the extract of the roots from in vitro-germinated plantlets

The roots from the two-month-old plantlets, which had been obtained by seeds collected in Tivoli Terme and grown under controlled conditions, contained 27 times more xanthone than the roots from the wild grown plants (11.65 ± 0.93 mg/g DW and 0.43 ± 0.03 μg/g DW, respectively). Moreover, the xanthone profiles (Figure 2) also differed: toxyloxanthone B was found, yet there were no traces of kielcorin (Table 1).

Table 1: Chemical analysis of root extracts obtained from in field-collected plants and grown under controlled conditions.

Compound	TivoliAPif*	PoliAPif*	TivoliRif*	PoliRif*	Riv*
			(mg/g DW)		
Chlorogenic acid	1.5 + 0.071	1.44+ 0.062	nd	nd	Nd
Vanillic Acid	nd	nd	0.002. ±0.002	nd	Traces
Quercetin	0.24 + 0.022	0.85+ 0.031	nd	nd	Nd
Quercetrin	1.612 + 0.064	1.21+ 0.071	nd	nd	Nd
Luteolin	0.036 + 0.002	0.021+ 0.004	Traces	0.025+ 0.002	Traces
Hyperosid	16.562 + 0.605	10.28+ 0.65	nd	nd	Nd
Rutin	0.334 + 0.013	0.142+ 0.035	nd	nd	Nd
Isoquercetin	4.851 ± 0.212	3.33± 0.048	nd	nd	Nd
1,3,5,6-; 1,3,6,7-tetrahydroxyxanthone	nd	nd	Traces	Traces	2.712±0.121
Kielcorin	nd	nd	0.173+ 0.011	0.036± 0.006	Nd
1,7-dihydroxanthones	nd	nd	0.011 + 0.005	0.039± 0.002	2.121±0.082
Toxyloxanthone B	nd	nd	0.073 + 0.003	nd	1.312±0.051
Paxanthone	nd	nd	0.171+ 0.013	nd	2.143±0.072
5-O-methyl-2-deprenylrheediaxanthone B	nd	nd	Traces	0.104± 0.012	3.274±0.154

*Aif, aerial parts from in field-grown plants; Rif, roots from in field-grown plants; Riv, roots from plants grown under controlled conditions. nd=not detected.

Antifungal activity

The antifungal activity of total root extracts from the wild grown plants and the two-month-old *in vitro* plantlets against *Candida albicans*, non-*albicans Candida* species, *C. neoformans*, and dermatophytes was evaluated (Table 2). The extract from the wild grown plant roots showed moderate antifungal activity against all of the tested fungi, with a geometric mean MIC of 663.98 μl/ml for *C. albicans*, 512 μl/ml for non-*albicans Candida* species, 215 μl/ml for dermatophytes and 53.81 μl/ml for *C. neoformans*. The extract from the roots of the plantlets grown *in vitro* showed greater antifungal that the extract from the root of wild grown plants, which is consisted with the higher amount of xanthones in the former. In particular, this extract showed greater antifungal activity against *C. albicans,* non *albicans Candida* species and dermatophytes, with a geometric mean of 73.94, 57.02, and 114 μl/ml, respectively; the antifungal activity against *Cryptococcus neoformans* was not greater than that found for the extract from wild grown roots.

Discussion

We evaluated the chemical profile of *H. perforatum* subsp *angustifolium* plants grown in the wild in different areas and grown under controlled conditions, and we investigated the pattern of xanthone accumulation and the antifungal activity of extracts containing xanthones. The interest in xanthones as new therapeutic agents lies in their documented microbiological activities (Fotie and Bohle, 2006). Pasqua et al. (2003) demonstrated that in *H. perforatum in vitro* systems, xanthones were accumulated in the roots regenerated from plantlets or formed by callus,

yet they were not found in the aerial parts. According to the literature, only traces of mangiferin have been found in the aerial parts of wild grown plants (Kitanov and Nedialkov, 1998).

Table 2: Antifungal activity against human fungal pathogen of root extracts obtained from in field-collected plants and grown under controlled conditions.
* Rif, roots from in field-grown plants; Riv, roots from plants grown under controlled conditions. **Geometric mean of the minimal inhibitory concentration (MIC). Strains: Candida albicans (ATCC10231; ATCC10261; ATCC3153; ATCC24433; ATCC90028; PMC 1011; PMC1002; PMC1010) Candida species no albicans (C .parapsilosis ATCC22019; C. parapsilosis DSM11224; C. tropicalis DSM11953; C tropicalis PMC0908, C. tropicalis PMC0910; C. glabrata PMC0805; C. krusei DSM6128, C. krusei PMC0613), Cryptococcus neoformans (DSM 11959, PMC2136, PMC2123, PMC2115, PMC2103, PMC2107, PMC2102, PMC2111), T. mentagrophytes DSM 4870, PMC6503, PMC6509, PMC6331, PMC6515, PMC6552; M. gypseum PMC7331, PMC7303).

Fungal strain	TivoliRif*		Riv*		Fluconazole	
	GM ** (μg/mL)	RANGE (μg/mL)	GM ** (μg/mL)	RANGE (μg/mL)	GM** (μg/mL)	RANGE (μg/mL)
Candida albicans	663.98	256-1024	73.94	32-128	1.3	0.25-16
Candida species	512	64-1024	57.02	16-128	2.31	0.5-64
Cryptococcus neoformans	53.81	32-128	53.82	32-64	2.06	0.5-4
dermatophytes	215.27	64-1024	114.03	32-1024	8	1-32

In the present study, no xanthones were detected in the aerial parts of the plants collected in either of the areas. In the root extracts of wild plants, the xanthones 1,7-dihydroxyxanthone, 5-O-methyl-2deprenylrheediaxanthone B, kielcorin, paxanthone, 1,3,5,6-

tetrahydroxyxanthone, and 1,3,6,7-tetrahydroxyxanthone were found. With regard to 1,7-dihydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone and paxanthone, to the best of our knowledge this is the first report of these xanthones in the roots of wild grown *H. perforatum* plants. In the roots collected in Tivoli Terme, kielcorin and paxanthone were the major xanthones, followed by 1,7-dihydroxyxanthone, whereas the 1,3,5,6- and 1,3,6,7-tetrahydroxyxanthones were only present in traces. By contrast, the major xanthone in the roots collected in Poli was 5-O-methyl-2-deprenylrheediaxanthone B, and only traces of paxanthone were. Although the total amount of xanthones detected was quite low (0.43± 0.03 and 0.18±0.02 mg/g DW in Tivoli Terme and Poli respectively), most of them have been found to have antimicrobial activity. 5-O-methyl-2-deprenylrheediaxanthone B has been shown to possess antifungal activity against species of the genus *Phomopsis* (Crockett el al., 2011). 1,7-dihydroxyxanthone and the 1,3,5,6- and 1,3,6,7-tetrahydroxyxanthones show antimicrobial activity against *Candida* spp strains (Fotie and Bohle, 2006). The differences found when comparing the wild grown plants in the two areas could be attributed to environmental factors such as, soil composition, and altitude. In light of the variability in metabolite content observed in the plants collected in different localities (Conforti et al., 2012), we investigated roots from plantlets grown under controlled conditions. Compared to the roots from the wild grown plants, those from the *in vitro* plantlets had a xanthone content that was 27 times greater; the roots from the plants also contained toxyloxanthone B, which was not found in the roots of wild grown plants, yet they did not contain kielcorin. A comparison of

antifungal activity of *H. perforatum* roots from wild grown plants and from plantlets grown under controlled conditions is reported for the first time in this study against *C. albicans*, non *albicans Candida* species, *C. neoformans*, and dermatophytes. The geometric mean MIC for the root extracts from the *in vitro* plantlets, compared to the MIC for the root extracts from the wild grown plants, was 9 times lower for *C. albicans*, 9 times lower for the non-*albicans Candida* species, and 2 times lower for dermatophytes. In conclusion, the roots of plants grown under controlled conditions have greater antifungal activity, especially against *Candida* spp, and this activity is positively correlated with xanthone accumulation**.** A future prospective study will include different species of *Hypericum* grown under controlled conditions, with the objective of identifying the species with the best antifungal activity.

Acknowledgements

This work was supported by a Grant C26A11XLZ5 of University "Sapienza" Rome, Italy and by the Project PON 01_01802.

References

1. Cervo L, Rozio M, Ekalle-Soppo CB, Guiso G, Morazzoni P, Caccia S. 2002. Role of hyperforin in the antidepressant-like activity of *Hypericum perforatum* extracts. Psychopharmacol 164(4):423-428.
2. Crockett SL, Poller B, Tabanca N, Pferschy-Wenzig EM, Kunert O, Wedge DE, Bucar F. 2011. Bioactive xanthones from

the roots of *Hypericum perforatum* (common St John's wort). J Sci Food Agric 91(3):428–434.

3. Dias ACP, Seabra RM, Andrade PB, Fernandes-Ferreira M. 1999. The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from in vivo and in vitro biomass of Hypericum species. J Liq Chromatogr Relat Technol 22:215-227.
4. Fenner R, Sortino M, Kuze Rates SM, Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nör C, von Poser G, Schapoval E, Zacchino S. 2005. Antifungal activity of some Brazilian *Hypericum* species. Phytomed 12:236-240.
5. Fotie J, Bohle DS. 2006. Pharmacological and biological activities of xanthones. Anti-Infective Agents Med Chem 5:15-31
6. Kartnig I, Gobel B, Heydel F. 1996. Production of hypericin, pseudohypericin and flavonoids in cell cultures of various *Hypericum* species and their chemotypes. Planta Med 62(1):51-3.
7. Kitanov GM, Nedialkov PT. 1998. Mangiferin and isomangiferin in some Hypericum species. Biochem. Syst Ecol 26: 647-653.
8. Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.
9. Pasqua G, Avato P, Monacelli B, Santamaria AR, Argentieri MP. 2003. Metabolites in cell suspension cultures, calli, and in vitro

regenerated organs of *Hypericum perforatum* cv. Topas. Plant Sci 165:977-982.

10. Pfaller MA, Diekema DJ. 2010. Epidemiology of invasive mycoses in North America. Cri Rev Microbiol 36:1–53.
11. Radulovic N, Stankov-Jovanovic V, Stojanovic G, Smelcerovic A, Spiteller M, Asakawa Y. 2007. Screening of in vitro antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans. Food Chem 103:15-21.
12. Rapp RP. 2004. Changing strategies for the management of invasive fungal infections. Pharmacotherapy 24:4–28.
13. Tocci N, D'Auria FD, Simonetti G, Panella S, Palamara AT, Pasqua G. 2012. A three-step culture system to increase the xanthone production and antifungal activity of Hypericum perforatum subsp. angustifolium in vitro roots. Plant Physiol Biochem 57: 54-58.
14. Tocci N, Simonetti G, D'Auria FD, Panella S, Palamara AT, Valletta A, Pasqua G. 2011. Root cultures of Hypericum perforatum subsp. angustifolium elicited with chitosan and production of xanthone-rich extracts with antifungal activity. Appl. Microbiol. Biotechnol. 91: 977-987.
15. Zobayed SMA, Murch SJ, Rupasinghe HPV, Saxena PK. 2004. *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). Plant Sci 166:333-340.

Annexes to the study not included in the paper

Annex 1
HPLC Methods

Table 1: Method 1*, gradient elution

Time (minutes)	Solution A (%)	Solution B (%)
1	90	10
2	70	30
8	60	40
13	50	50
15	50	50
23	30	70
28	30	70
38	20	80
50	20	80
55	5	95
65	5	95
70	10	90

*The method is a modification of the one reported by Dias et al. 1999.

Table 2: Method 2*, isocratic elution

Acetonitrile (%)	Water (%)	Phosphoroic acid (%)
16	83	1

*Kartig et al. (1996)

Annex 2
Standard compounds

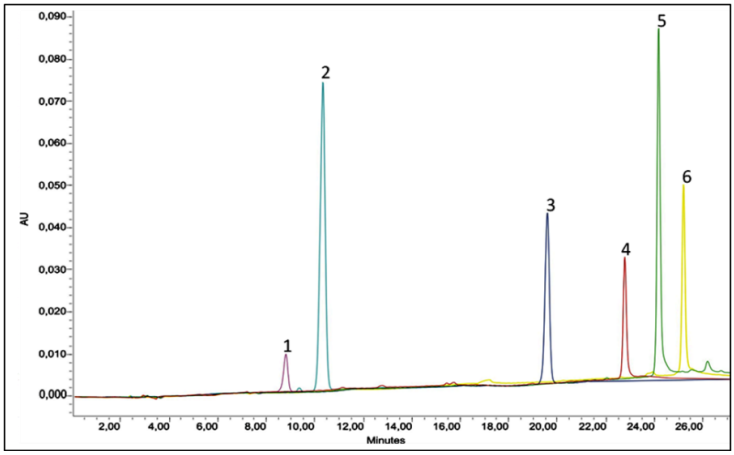


Fig.1 HPLC chromatogram of standard compounds: 1, chlorogenic acid; 2, vanillic acid; 3 quercetrin; 4, quercetin; 5, luteolin; 6, kampferol.

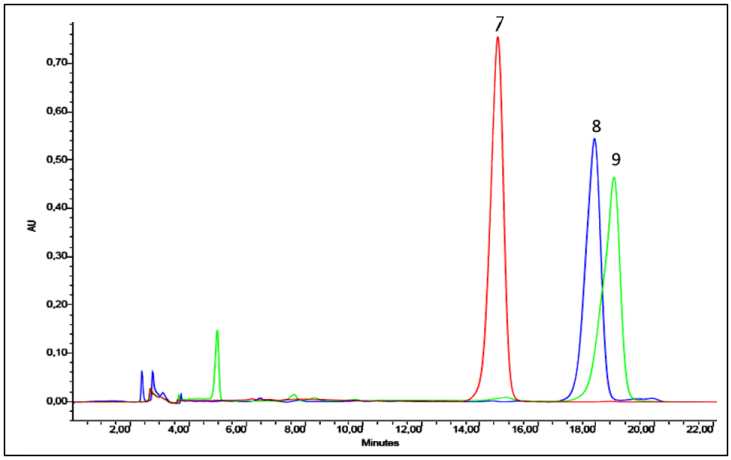


Fig.2 HPLC chromatogram of standard compounds: 7, rutin; 8, hyperoside; 9, isoquercetrin.

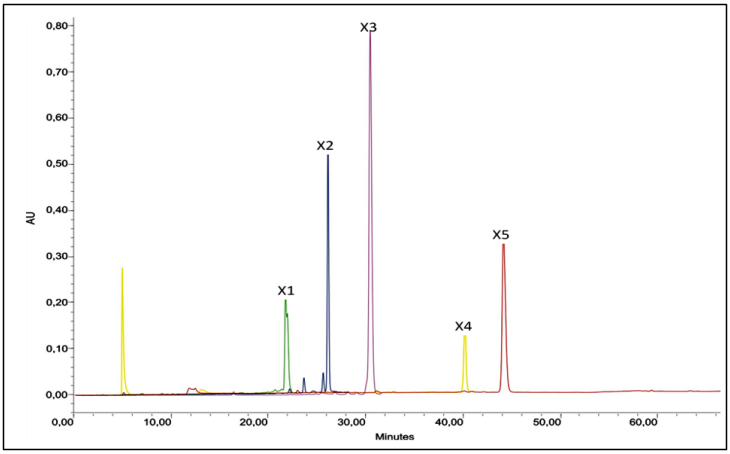


Fig.3 HPLC chromatogram of xanthone reference compounds: X1, 1,3,6,7-, 1,3,5,6,- tetrahydroxyxanthone mix; X2, kielcorin; X3, 1,7-dihydroxyxanthone; X4,paxanthone; X5, 5-O-methyl-2-deprenylrheediaxanthone B

Annex 3
Calibration curves

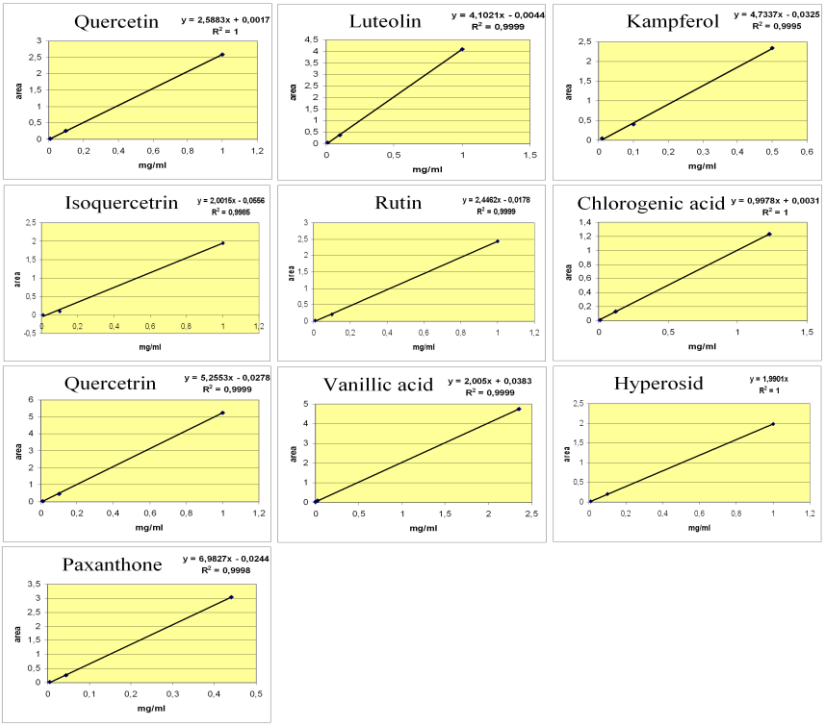


Fig.1 Calibration curves for standards compounds used to quantitatively analyze the extract composition.

Chapter 4:

From the plant to *in vitro* cultures

The chemical analysis performed on wild grown *H. perforatum* plants demonstrated that roots are the accumulation organ of xanthones. However the detected amount was very low and sensitive to environmental factors. These limitations lead to the establishment of cultures of plants grown under controlled conditions to investigate the potential of *H.perforatum* root extracts for applicative purposes. The extract from roots of plants grown under controlled conditions accumulated higher amounts of xanthones and the antifungal activity was improved.

It is known that root cultures are a good system to produce secondary metabolites. In the light of the data obtained from *in vivo* plants, roots have been regenerated from callus of Hpa and cultures have been established and analyzed to detect xanthone accumulation.

Chapter 5:

Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity

Paper published in Applied Microbiology and Biotechnology
DOI 10.1007/s00253-011-3303-6
Received: 4 February 2011 / Revised: 30 March 2011 / Accepted: 1 April 2011

Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity

Noemi Tocci[1], Giovanna Simonetti[2], Felicia Diodata D'Auria[2], Simona Panella[2], Anna Teresa Palamara[2], Alessio Valletta[1], Pasqua Gabriella[1*]

[1]Department of Environmental Biology, [2]Department of Public Health and Infectious Diseases "Sapienza" University of Rome; Piazzale Aldo Moro 5; 00185 Rome, Italy
*The authors Noemi Tocci and Giovanna Simonetti contributed equally to this work

Abstract
Hypericum perforatum is a well known medicinal plant which contains a wide variety of metabolites, including xanthones, which have a wide range of biological properties, including antifungal activity. In the present study, we evaluated the capability of roots regenerated from calli of *Hypericum perforatum* subsp. *angustifolium* to produce xanthones. Root biomass was positively correlated with the indole-3-butyric acid concentration, whereas a concentration of 1 mg l^{-1} was the most suitable for development of roots. High auxin concentrations also inhibited xanthone accumulation. Xanthones were produced in large amounts,

with a very stable trend throughout the culture period. When the roots were treated with chitosan, the xanthone content dramatically increased, peaking after 7 days. Chitosan also induced a release of these metabolites into the culture. The maximum accumulation (14.26 ± 0.62 mg g^{-1} dry weight) and release (2.64±0.13 mg g^{-1} dry weight) of xanthones were recorded 7 days after treatment. The most represented xanthones were isolated, purified and spectroscopically characterized. Antifungal activity of the total root extracts was tested against a broad panel of human fungal pathogen strains (30 *Candida* species, 12 *Cryptococcus neoformans,* and 16 dermatophytes); this activity significantly increased when using chitosan. Extracts obtained after 7 days of chitosan treatment showed high antifungal activity (mean MIC 83.4 μg ml^{-1}, of 39.1 μg ml^{-1}, and 114 μg ml^{-1}, against, respectively, *Candida* spp, *C. neoformans*, and dermatophytes). Our results suggest that root cultures can be considered as a potential tool for large-scale production of extracts with stable quantities of xanthones.

Keywords *Hypericum perforatum*, root cultures, xanthones, *Candida* spp., *Cryptococcus neoformans,* dermatophytes

Introduction

The pharmacological properties of *Hypericum perforatum* are well known. It contains a wide variety of metabolites with documented biological activities, including phenolic acids, flavonoids, naphthodianthrones, phloroglucinols and xanthones (Nahrstedt and Butterweck 2010). In a preliminary study on naturally grown plants of *H. perforatum* subsp.

angustifolium (HPA) (unpublished), we found that xanthones were mainly produced in the roots, though in very low quantities, whereas flavonoids, naphtodianthrones and phloroglucinols were distributed in the aerial parts of the plant.

Xanthones have a wide range of biological and pharmacological properties, such as monoamine oxidase inhibition, and antioxidant, antimicrobial, cytotoxic, and hepatoprotective activity (Fotie and Bohle 2006). The antifungal activity of many xanthones has also been well documented (Lavault et al. 2005; Wang et al. 2005; Pinto et al. 2011). This reflects the renewed interest in plant extracts with antifungal activities, which has been due to the increasing number of immunocompromised persons (e.g., organ transplant recipients, chemotherapy patients, and persons with AIDS), and the consequent sharp increase in the frequency of fungal infections (Pfaller and Diekema, 2010). This in turn has led to an increase in the prescription of antifungal drugs and the emergence of drug resistance. In light of these circumstances and considering that there is only a limited arsenal of antifungal drugs and agents, new therapeutic approaches must be developed (Rapp 2004; Pfaller et al. 2010). Although the antifungal activity of xanthones from a number of *Hypericum* species against human pathogenic fungi has been investigated (Fenner et al. 2005; Radulovic et al. 2007), little is known about the possible application of xanthones from *H. perforatum* roots.

The plant kingdom has historically been a major source of bioactive compounds for medicine, food additives, pigments, insecticides, cosmetics, and fine chemicals. However, the recovery of bioactive

compounds from natural sources is often problematic, in that the extract may contain only very small quantities or its composition may vary with the season or the environment. To obtain qualitatively and quantitatively standardized extracts, plant biotechnology can represent a valuable alternative.

In the present study, we evaluated the capability of regenerated roots from calli of HPA to produce xanthones, which has never been investigated. To increase xanthone production, we used chitosan, an effective biotic elicitor for improving xanthone biosynthesis in cell cultures of HPA (Tocci et al. 2010). We also tested the antifungal activity of total extracts and of the most representative xanthones against a broad panel of human fungal pathogens.

Materials and methods

Plant material and tissue culture

Seeds of HPA were surface sterilized in accordance with Zobayed et al. (2004) and placed on Murashige and Skoog (MS) (1962) medium (Duchefa Biochemie, Netherlands) supplemented with 3% (w/v) sucrose and solidified with agar. Seed cultures were grown under a photoperiod of 16/8 h (light/dark) at 26°C (photon lux density 70 μmol m^{-2} s^{-1}). To induce the regeneration of adventitious roots, leaf and stem explants were excised from 2-month-old seedlings and placed on solid MS medium supplemented with 5 mg l^{-1}, 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, Netherlands), 1 mg l^{-1} kinetin (Duchefa Biochemie, Netherlands), and 3% (w/v) sucrose; the cultures were maintained under continuous darkness. After 28 days of culture, the callus were transferred to hormone-free (HF) agarized MS medium, to

induce the formation of roots. Regenerated roots, 2-3 cm in length, were isolated from the callus (after 30 days) and subcultured in the same medium. To induce branching (lateral root formation) and to increase biomass, the roots (after 30 days) were cultured in MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) (0, 0.5, 1, 1.5 and 2 mg l^{-1}). Liquid cultures were established after 30 days, by inoculating 0.02 g dry weight (DW) of roots in 250 ml Erlenmeyer flasks containing 100 ml half-strength liquid MS medium supplemented with 1 mg l^{-1} IBA. The flasks were shaken at 100 rpm at 25±1 C° and maintained in the dark. Subcultures in liquid medium were performed every 23 days.

Elicitation

Xanthones were elicited using chitosan (medium molecular weight, Sigma-Aldrich, Italy) at a final concentration of 200 mg l^{-1}, which was added on the 8th day of culture. Root samples were harvested by filtration on the 11th, 15th, 18th and 23rd days of culture.

Determination of root biomass

The biomass was determined by recording the DW of the roots. The roots were separated from the medium by passing them through a stainless steel sieve and washed with distilled water to remove medium contaminants. They were then dried in an oven at 40°C for 72 hours, and the DW was measured. The growth index was calculated as follows:

$$\text{Growth Index} = \frac{\text{Final dried weight - Initial dried weight}}{\text{Initial dried weight}}$$

HPLC analysis

Ground-dried roots (4-6 months in culture) were extracted three times with methanol, at room temperature. The culture media were extracted with ethyl acetate using a separating funnel. The final volume of the extracts was dried with a rotavapor and subjected to HPLC. The HPLC analysis of the extracts was performed using an apparatus consisting of a pump (Waters 1525 Binary HPLC Pump), equipped with a UV detector (Waters 2487 Dual λ Detector) and a reverse phase C_{18} column (4.6 mm X 150 mm; 5 μm; Waters). The extract composition was determined by linear gradient elution, using a modified version of the method of Dias et al. (1999). The mobile phase was a gradient prepared from 100:0.1 (*v*/*v*) water–phosphoric acid (component A) and methanol (component B) (Carlo Erba, Milan, Italy); the gradient program was from 10% to 50% B in 13 min, hold for 10 min, and then from 50% to 95% B in 42 min. The injection volume was 20 μl, the mobile phase flow rate was 1.0 ml min^{-1}. The compounds were identified at 260 nm and 320 nm by the external standard method, using, as reference, xanthones that had been previously purified and spectroscopically characterized in our laboratory (1,3,6,7-tetrahydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone, toxyloxanthone B, paxanthone, and cadensin G). 1,7-dihydroxyxanthone and 5-methoxy-2-deprenylrheediaxanthone B were purified from in vitro regenerated roots of HPA. Xanthones were quantified as paxanthone equivalents at 260 nm.

Purification and ^{1}H-NMR analysis of xanthones

The isolation and purification of 1,7-dihydroxyxanthone and 5-methoxy-2-deprenylrheediaxanthone B were carried out by preparative thin layer

chromatography (silica gel, hexane:ethyl acetate 2/1) (Carlo Erba, Milan, Italy). The nuclear magnetic resonance spectroscopic data were recorded at room temperature on an Avance 400MHz NMR Spectrometer. The NMR chemical shifts (δ) are expressed as parts per million (ppm) relative to the residual proton chemical shifts of the deuterated solvent (acetone-D6) set relative to external tetramethylsilan (TMS).

1,7-dihydroxyxanthone (euxanthone): ^{1}H-NMR (400 MHz, AcD_6): δ 12.72 (1H, s, OH–C1), 7.72 (1H, t, J= 8.0 Hz, H-3), 7.63 (1H, d, J= 2.8 Hz, H-8), 7.54 (1H, d, J= 9.2 Hz, H-5), 7.45 (1H, dd, J= 9.2 and 2.8 Hz, H-6), 7.02 (1H, d, J= 8.0 Hz, H-4), 6.78 (1H, d, J= 8 Hz, H-2).

5-methoxy-2-deprenylrheediaxanthone: B ^{1}H-NMR (400 MHz, AcD_6) δ: 13.40 (OH-1, s), 7.85 (H-8, d, J = 8.7 Hz), 7.03 (H-7, d, J = 8.7 Hz), 6.18 (H-2, s), 4.60 (H-2', q, J = 6.6 Hz), 4.02 (OMe, s), 1.65 (4'-CH_3 , s), 1.43 (3'-CH_3, d, J= 6.6 Hz), 1.35 (5'- CH_3, s).

Organisms

For the antifungal evaluation, strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany) and from the Pharmaceutical Microbiology culture collection (PMC, Department of Public Health and Infectious Diseases, Sapienza, Rome, Italy) were tested. The strains obtained from the ATCC were: *Candida albicans* ATCC90028, *C. albicans* ATCC90029, *C. albicans* ATCC10261, *C. albicans* ATCC10231, *C. albicans* ATCC3153, *C. albicans* ATCC2091, *C. albicans* ATCC76615, *C. albicans* ATCC24433 (tested as reference strain), *Candida parapsilosis* ATCC22019 (tested as quality control strain), and *Trichophyton mentagrophytes* ATCC9972 (tested as reference strain). The

strains obtained from the DSMZ were: *C. parapsilosis* DSM11224, *C. parapsilosis* DSM5784, *Candida krusei* DSM6128, *Candida tropicalis* DSM11953, *Candida glabrata* DSM11226, *Cryptococcus neoformans* DSM11959, *C. neoformans* DSM6972, *T. mentagrophytes* DSM4870, *Trichophyton rubrum* DSM4167, *Microsporum canis* DSM10708, *Microsporum gypseum* DSM 3824. The strains obtained from PMC were: *C. albicans* (PMC 1011, PMC 1015, PMC 1023, PMC 1071, PMC 1075, PMC 1083, PMC 1088, PMC 1097), *C. parapsilosis* (PMC 0703, PMC 0711), *C. tropicalis* PMC 0908, *C. krusei* (PMC 0613, PMC 0625, PMC 0639), *C. glabrata* (PMC 0805, PMC 0821, PMC 0843), *C. neoformans* (PMC 2103, PMC 2107, PMC 2111, PMC 2123, PMC 2136, PMC 2138, PMC 2142, PMC 2157, PMC 2169, PMC 2185), *T. mentagrophytes* (PMC 6503, PMC 6509, PMC 6515, PMC 6527, PMC 6531, PMC 6552), *T. rubrum* PMC 6612, *M. gypseum* (PMC 7303, PMC 7331, PMC 7342), *M. canis* PMC 7426. All the strains were stored at -70°C in 10-15% glycerol solution (CLSI 2008a; CLSI 2008b). Yeasts were grown on sabouraud dextrose agar (Sigma-Aldrich, St. Louis, Missouri, U.S.A) plate for 24-48h at 35°C, in accordance with the procedures of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2008a). Dermatophytes were grown on potato dextrose agar (Sigma-Aldrich, St. Louis, Missouri, U.S.A) at 30°C for 4-5 days or until good conidial growth was obtained (CLSI 2008b).

Antifungal susceptibility testing

In vitro antifungal susceptibility was evaluated using the total extracts from untreated and treated roots obtained on the 15th day of culture, and from isolated and purified xanthones. The tests were conducted in accordance with the CLSI M27-A3 and CLSI M38-A2 broth

microdilution methods (CLSI 2008a; CLSI 2008b). Yeast and dermatophyte inocula were prepared as described in the CLSI protocols. The antifungal references amphotericin B and fluconazole (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) were also tested (ranges: 0.031 to 16 μg ml^{-1} for amphotericin B and 0.0078 to 250 μg ml^{-1} for fluconazole). The final concentrations ranged from 1000 to 1 μg ml^{-1} for total extracts and from 64 to 0.125 μg ml^{-1} for single xanthones. Microdilution trays containing 100 μl of serial two-fold dilutions of each substance in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, U.S.A) were inoculated with an organism suspension adjusted to attain a final inoculum concentration of 1.0 x 10^3 - 1.5 x10^3 cells ml^{-1} for yeasts and 0.4x10^4 - 5x10^4 CFU ml^{-1} for dermatophytes. The panels were incubated at 35°C and observed for the presence of growth at 48 h (*Candida* spp.) and 72 h (*C. neoformans* and dermatophytes). Quality control was performed by testing the CLSI-recommended strain *C. parapsilosis* ATCC 22019 (CLSI 2008c). The minimum inhibitory concentration (MIC) was defined as, for yeasts, the lowest concentration that showed ≥50% growth inhibition (MIC_{50}) compared with the growth control and, for dermatophytes, the lowest concentration that showed ≥80% reduction in growth (MIC_{80}) with respect to the growth control. However, when testing strains against amphotericin B, the MIC was defined as the lowest concentration that prevented any discernible growth (MIC_{100}). The MIC_{100} was also evaluated for all of the substances. All of the experiments were performed in triplicate on three consecutive test days.

Cell viability assay

Human leukemic monocyte lymphoma cells (U937) were obtained from the American Type Culture Collection (ATCC CRL1593.2,- Rockville, MD, U.S.A.). The cells ($2x10^4$ cells $well^{-1}$) were seeded into 96-well plates containing 100 µl of supplemented RPMI1640 (Invitrogen, San Diego, CA, U.S.A.) without phenol red, supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA, U.S.A.), L-glutamine (0.3 mg ml^{-1}), penicillin (100U ml^{-1}) and streptomycin (100 µg ml^{-1}) (EuroClone-Celbio, Milano, Italy), and they were cultured at 37°C in 5% CO_2. Total extracts, dissolved in dimethyl sufoxide (DMSO), from untreated and treated roots obtained on the 15th day of culture (with concentrations ranging from 64 to 1000µg ml^{-1}) were added to the wells. Each concentration and control was assayed in four replicates with at least five concentrations. The cells were cultured at 37° C and 5% CO_2 for 24 h, and cell viability was determined using an MTT-assay (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) (Mosmann 1983). MTT solution was added to each well in an amount equal to 10% of the culture volume, and the plates were incubated for 3-4 h at 37°C in 5% CO_2. MTT solvent (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was successively added to dissolve the intracellular crystal. The plates were then incubated at 37° C in 5% CO_2 for 1 h, and the optical density of each well was measured spectrophotometrically at 570 nm. The cytotoxicity of the root extracts was calculated as the percentage reduction in viable cells with respect to the control culture (cells treated with DMSO only). The 50% cytotoxic concentration (CC_{50}) was evaluated as the drug concentration required to reduce human cell viability by 50% compared to the drug-free control.

Statistical analysis

The arithmetic mean and Standard Deviation (SD) were calculated. The significance of the differences between the means was tested by a two-tailed Student's *t*-test. $P<0.05$ was considered to be significant; $P<0.001$ was considered to be highly significant.

Results

Induction and development of adventitious roots

The seeds of HPA germinated within 2 days. On the 8th day of culture, cotyledons were evident. Leaf and stem explants from 2-month-old seedlings were inoculated in a callogenic medium. After 28 days of culture, the callus, which was cream colored and friable, was transferred to HF medium, to induce root regeneration. Twenty days later, regenerated roots were visible. When roots reached a length of 2-3 cm, they were separated from the callus and subcultured in the same medium. Their growth in length was initially very slow, but they grew rapidly after about 30 days of culture. The roots showed regular morphology and structure.

The effect of IBA on biomass growth and xanthone production

To achieve the most suitable culture conditions, the roots were cultivated in MS liquid medium supplemented with different concentrations of IBA (0, 0.5, 1, 1.5 and 2 mg l^{-1}). Root biomass increase was monitored for 23 days based on the growth curve (Fig. 3a). In this system the log phase ended on the 18th day of culture and no additional increase in biomass was observed until the 23rd day. Concerning biomass

increase, the highest growth indexes (8.5 and 9.5) were registered for roots cultivated in the presence of the highest IBA concentrations (Fig.1) (1.5 and 2 mg l^{-1}), yet in these conditions the roots showed callus formation. In the medium supplemented with 1 mg l^{-1} IBA, the roots showed a growth index of 6.5, which corresponded to a 7.5 increase in biomass with respect to the initial inoculum. Even if the biomass increase with IBA 1.5 and 2 mg l^{-1} was significantly higher ($P<0.05$) than with IBA 1 mg l^{-1}, this last medium has been chosen because it allowed the formation of morphologically unaltered roots. This medium was also found to have been the most suitable to induce a significant higher ($P<0.05$) xanthone accumulation (Fig. 2) (3.26 ± 0.09 mg g^{-1} DW).

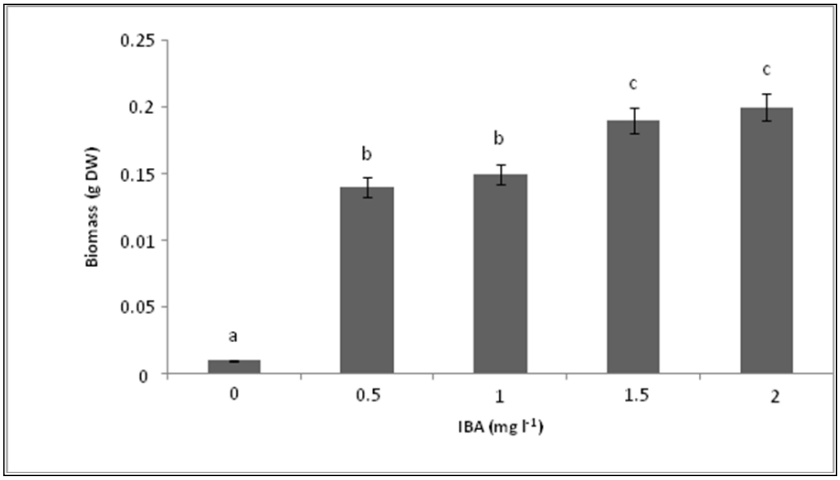


Figure 1 Biomass increase (g DW) for roots cultivated in MS medium supplemented with different concentrations of IBA (0; 0.5; 1; 1.5; 2 mg l^{-1}). Results are means (±SD) of three independent replicates. Different letters refer to significant differences ($P<0.05$).

Effect of chitosan treatment on root biomass growth

The growth curve of root biomass was recorded during a culture cycle of 23 days (Fig. 3a). Chitosan, which was added during the exponential phase (8th day of culture), resulted in a cessation in growth and a decrease in biomass.

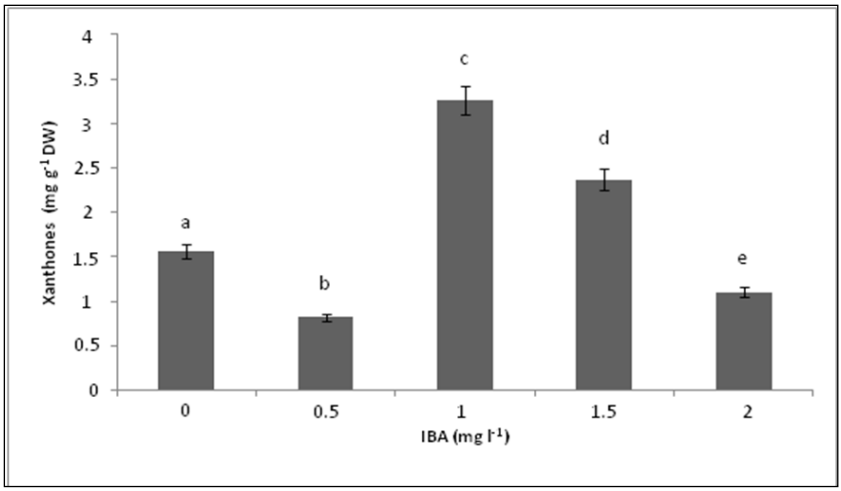


Figure 2 Total xanthone accumulation (mg gDW^{-1}) in roots cultivated in MS medium supplemented with different concentrations of IBA (0; 0.5; 1; 1.5; 2 mg l^{-1}). Results are means (±SD) of three independent replicates. Different letters refer to significant differences ($P<0.05$).

Xanthone production in untreated and chitosan-treated roots

The HPLC analysis showed that chitosan treatment greatly, though only transiently, enhanced xanthone production. Xanthone accumulation in untreated roots did not vary dramatically during the culture period (Fig. 3b), with a mean of 4.5± 1.5 mg g^{-1} DW.

The major xanthones accumulated in the untreated roots were 1,7-dihydroxyxanthone, 5-methoxy-2-deprenylrheediaxanthone B, and paxanthone. 1,7-dihydroxyxanthone was detected from the beginning of the culture period until the 15th day, whereas afterwards it was not detected (Table 1). The xanthone profile in treated root extracts was qualitatively similar to the one in untreated root extracts. In treated roots, total xanthone accumulation peaked 7 days after treatment (which corresponded to the 15th day of culture) at 14.26±0.62 mg g^{-1} DW, which is significantly higher ($P<0.05$) than the accumulation in the control on the same day of culture.

Afterwards, total xanthone accumulation rapidly decreased, until reaching 3.01±0.09 mg g^{-1} DW on the 23rd day of culture, which is similar to that for untreated roots (3.26±0.09 mg g^{-1} DW) (Fig. 3b). Moreover, the peak accumulation of 1,7-dihydroxyxanthone in treated roots (7 days after treatment) was 10-times higher than the accumulation in untreated roots.

Xanthone release in the culture medium

The HPLC analysis of the culture media of treated and untreated roots showed qualitatively similar chemical profiles for xanthones, with 1,7-dihydroxyxanthone being the main compound in all of the extracts (Table 2). Total xanthone release was significantly enhanced by chitosan ($P<0.05$), peaking on the 15th day of culture (7 days after treatment) at 2.64±0.13 mg g^{-1} DW (21.12 mg l^{-1} medium); 1.7±0.1 mg g^{-1} DW (13.6 mg l^{-1} medium) of the total xanthone release was represented by 1,7-dihydroxyxanthone.

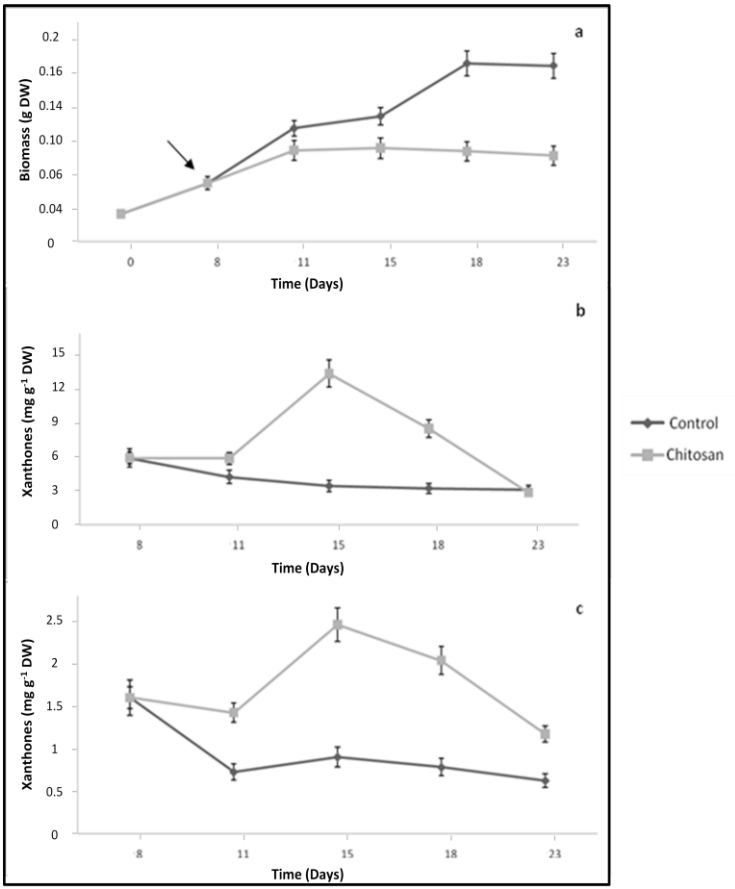


Figure 3 (a) Growth curves of untreated and chitosan-treated root cultures; (b) accumulation and (c) release of detected xanthones by root cultures of HPA, throughout the growth period. Results are means (±SD) of three independent replicates. The straight arrow in (a) indicates the day of chitosan treatment.

Antifungal activity

The antifungal activity of the extracts obtained from untreated and treated roots at the 15th day of culture was evaluated against strains of *Candida* spp., *C. neoformans* and dermatophytes. Chitosan increased the antifungal activity against all of the strains tested. In particular, for the treated roots, the MIC range against *Candida* spp. strains was 16-250 μg ml^{-1}, compared to 64-1000 μg ml^{-1} for untreated roots (Table 3). Regarding the extracts from treated roots, when comparing strains of *Candida* non-*albicans* species with *C. albicans*, the MIC range was, respectively, 16-250 μg ml^{-1} and 64-250 μg ml^{-1} (Table S1 in the electronic supplementary material). For strains of *C. neoformans*, the MIC range was 16-125 μg ml^{-1} for treated-root extracts and 64-250 μg ml^{-1} for untreated-root extracts. For dermatophyte strains, the MIC range was 64-125 μg ml^{-1} and 125-500 μg ml^{-1} for treated-root and untreated-root extracts, respectively (Tables 4 and 5) (additional data are given in on line resource Tables S2 and S3).

The difference in MIC between treated and untreated roots was highly significant (two-tailed Student's *t*-test: $P<0.001$) for *Candida* and *Cryptococcus* strains (Tables 3 and 4) and significant ($P<0.05$) for dermatophytes (Table 5). Total growth inhibition (MIC_{100}) for all tested strains was obtained with concentrations ranging from about 125 to 1000 μg ml^{-1}. The main xanthones isolated and purified from treated-root extracts (1,7-dihydroxyxanthone, cadensin G, paxanthone, and 5-methoxy-2-deprenylrheediaxanthone B) were also investigated for their antifungal activity; the MIC ranged from 4 to > 64 μg ml^{-1} (Tables 3, 4,

and 5) (additional data are given in on line resource Tables S1, S2 and S3). 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone have not been tested because, no one of the solvents used allowed their separation due to their structural similarity.

Table 1 Xanthones detected in extracts of untreated (-) and chitosan-treated (+) roots.

	Day 8		Day 11		Day 15		Day 18		Day 23	
Xanthones	**- mg g^{-1} DW**	**+ mg g^{-1} DW**	**- mg g^{-1} DW**	**+ mg g^{-1} DW**	**- mg g^{-1} DW**	**+ mg g^{-1} DW**	**- mg g^{-1} DW**	**+ mg g^{-1} DW**	**- mg g^{-1} DW**	**+ mg g^{-1} DW**
1, 2	nd	nd	nd	0.75 ±0.02	0.37 ±0.01	1.2 ±0.04	0.35 ±0.01	0.88 ±0.03	0.32 ±0.01	nd
3	1.93 ±0.09	1.93 ±0.09	1.5 ±0.04	1.18 ±0.03	0.83 ±0.02	8.36 ±0.39	nd	5.04 ±0.23	nd	0.69 ±0.02
4	2.04 ±0.1	2.04 ±0.01	1.5 ±0.05	1.18 ±0.04	0.83 ±0.02	nd	0.66 ±0.02	Nd	0.52 ±0.01	0.56 ±0.02
5	0.74 ±0.02	0.74 ±0.02	0.44 ±0.01	1.16 ±0.02	0.35 ±0.01	2.48 ±0.12	0.83 ±0.03	1.12 ±0.01	0.75 ±0.02	0.54 ±0.02
6	0.85 ±0.03	0.85 ±0.03	0.66 ±0.01	0.78 ±0.01	0.53 ±0.02	1.44 ±0.05	0.62 ±0.01	1.57 ±0.02	0.83 ±0.03	0.64 ±0.02
7	0.71 ±0.01	0.71 ±0.01	0.39 ±0.01	1.2 ±0.02	0.73 ±0.03	0.78 ±0.02	0.96 ±0.03	0.46 ±0.01	0.84 ±0.02	0.58 ±0.01
Total	6.21 ±0.21	6.28 ±0.21	4.49 ±0.12	6.25 ±0.14*	3.64 ±0.11	14.26 ±0.62*	3.42 ±0.10	9.07 ±0.30*	3.26 ±0.09	3.01 ±0.09

The data are the means ± standard error of three replicates. (1,2) 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone (it is a mixture of both); (3) 1,7-dihydroxyxanthone; (4) cadensin G; (5) toxyloxanthone B; (6) paxanthone; (7) 5-O-methyl-2-deprenylrheediaxanthone B. DW=dry weight; nd= not detected. *, $P<0.05$ (versus control roots, by Student's *t*-test).

Table 2 Xanthones detected in culture media of untreated (-) and chitosan-treated (+) roots.

	Day 8		Day 11		Day 15		Day 18		Day 23	
Xanthones	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**
	mg g^{-1} DW	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**	**mg g^{-1} DW**
1, 2	nd	nd	nd	0.4 ±0.01	nd	0.37 ±0.01	nd	nd	nd	nd
3	1.06 ±0.08	1.06 ±0.08	0.44 ±0.01	0.42 ±0.01	0.33 ±0.01	1.7 ±0.1	nd	1.35 ±0.15	nd	0.54 ±0.02
4	nd	nd	nd	0.37 ±0.01	nd	nd	0.21 ±0.01	0.42 ±0.02	0.12 ±0.01	0.72 ±0.02
5	nd	nd	nd	0.34 ±0.01	0.32 ±0.01	nd	0.20 ±0.01	0.42 ±0.02	0.18 ±0.01	Traces
6	nd	nd	nd	nd	nd	0.31 ±0.01	0.21 ±0.01	nd	0.23 ±0.01	Traces
7	0.66 ±0.01	0.66 ±0.01	0.34 ±0.01	nd	032 ±0.01	0.26 ±0.01	0.22 ±0.01	nd	0.14 ±0.01	nd
Total	1.72 ±0.09	1.72 ±0.09	0.78 ±0.02	1.53 ±0.04*	0.97 ±0.03	2.64 ±0.13*	0.84 ±0.04	2.19 ±0.19*	0.67 ±0.04	1.26 ±0.04*

The data are the means ± standard error of three replicates. (1,2) 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone (it is a mixture of both); (3) 1,7-dihydroxyxanthone; (4) cadensin G; (5) toxyloxanthone B; (6) paxanthone; (7) 5-O-methyl-2-deprenylrheediaxanthone B. DW=dry weight; nd= not detected. *, $P<0.05$ (versus control roots, by Student's *t*-test)

Table 3 Antifungal activity of total extracts from untreated and chitosan-treated roots of HPA obtained on the 15th day of culture and isolated and of purified xanthones against 30 *Candida* strains (16 *C. albicans*, 4 *C. parapsilosis*, 2 *C. tropicalis*, 4 *C. krusei*, 4 *C. glabrata*)

	MIC±SD (μg ml^{-1})	**MIC range (μg ml^{-1})**	**MIC$_{100}$ (μg ml^{-1})**	**QC strain MIC (μg ml^{-1})**
Control roots	281.2±198	64-1000	838.5±267	125
Treated roots	83.4±52.4*	16-250	489.6±260	32
3	>64	>64	>64	16
4	>64	>64	>64	32
6	>64	>64	>64	64
7	>64	>64	>64	8
Amphotericin B	n.d.	0.125-16	2.68±3.8	0.5
Fluconazole	2.57±4.06	0.125-16	95.5±122	2

*(3) 1,7-dihydroxyxanthone; (4) cadensin G; (6) paxanthone; (7) 5-O-methyl-2-deprenylrheediaxanthone B. MIC=arithmetic mean of minimum inhibitory concentration; SD= standard deviation; MIC$_{100}$=lowest drug concentration that prevented any discernible growth with respect to the untreated control; QC=quality control strain; data represent mean of three separate experiments in triplicate. n.d.=not determined. *, $P<0.001$ (versus control roots, by Student's *t*-test)

Table 4 Antifungal activity of total extracts from untreated and chitosan-treated roots of HPA obtained on the 15th day of culture and isolated and of purified xanthones against 12 strains of *C. neoformans*.

	MIC±SD (μg ml^{-1})	**MIC range** (μg ml^{-1})	**MIC_{100}** (μg ml^{-1})
Control roots	99±55.5	64-250	333±119
Treated roots	39.1±19*	16-125	146±46
3	>64	>64	>64
4	44± 15.6	32-64	>64
6	>64	>64	>64
7	42±15	32-64	>64
Amphotericin B	n.d.	0.016-4	1.63±1.55
Fluconazole	2.23± 1.57	0.5-8	15.1±17.7

*(3) 1,7-dihydroxyxanthone; (4) cadensin G; (6) paxanthone; (7) 5-O-methyl-2-deprenylrheediaxanthone B. MIC=arithmetic mean of minimum inhibitory concentration; SD= standard deviation; MIC_{100}=lowest drug concentration that prevented any discernible growth with respect to the untreated control; QC=quality control strain; data represent mean of three separate experiments in triplicate. n.d.=not determined. *, $P<0.001$ (versus control roots, by Student's *t*-test)

Table 5 Antifungal activity of total extracts from untreated and chitosan-treated roots of HPA obtained on the 15th day of culture and isolated and of purified xanthones against 16 dermatophytes (8 *T. mentagrophytes*, 2 *T. rubrum*, 4 *M. gypseum*, 2 *M. canis*).

	MIC±SD (μg ml^{-1})	MIC range (μg ml^{-1})	MIC_{100} (μg ml^{-1})
Control roots	321±164	125-500	642±305
Treated roots	114±235*	64-125	229±47
3	34±20	4-64	>64
4	>64	>64	>64
6	>64	>64	>64
7	46±17	16-64	>64
Amphotericin B	n.d.	0.125-8	4.8±3.1
Fluconazole	11.6±10.4	1-32	39±37.5

(3)1,7-dihydroxyxanthone; (4)cadensin G; (6)paxanthone; (7)5-O-methyl-deprenylrheediaxanthone B. MIC=arithmetic mean of minimum inhibitory concentration, SD=standard deviation; MIC_{100}=lowest drug concentration that prevents any discernible growth with respect to the untreated control; data represent mean of three separate experiments in triplicate. n.d.=not determined. *, $P<0.05$ (versus control roots, by Student's *t*-test)

Cytotoxic activity

The cytotoxicity of extracts obtained from untreated and treated roots at the 15th day of culture was evaluated against mammalian cells (U937). The CC_{50} was calculated. For both untreated and treated root extracts, the CC_{50} was >1000 μg ml^{-1} .

Discussion

In a comparative study of *H. perforatum* subspecies, using in-field grown plants, the accumulation of secondary metabolites was greater for HPA, compared to the other subspecies (Males et al. 2006). Xanthones with interesting biological activities have been found in *H. perforatum* total plant extracts (Kitanov and Blinova 1987; Holzl et al. 1989; Nahrstedt and Butterweck 1997). In an in vitro study of *H. perforatum*, Pasqua et al.

(2003) demonstrated that xanthones were accumulated in the roots regenerated from plantlets or formed by callus. These results were confirmed in an unpublished study on HPA, in which we found that in naturally grown plants xanthones were mainly accumulated in the roots, yet in very low quantities, ranging from 200 to 500 μg g^{-1} DW (unpublished). We also observed that xanthone accumulation greatly depended on the season, the soil composition, and the developmental stage of the plant. For these reasons, it is very difficult to recover an exploitable and standardized extract containing xanthones from natural sources.

To overcome these problems, we focused on in vitro regenerated root cultures as a biotechnological system for obtaining standardized extracts containing xanthones. The large-scale cultivation of untransformed roots for producing important phytochemicals has been achieved for medicinal plants such as *Nothapodytes foetida* (Fulzele et al. 2002), *Panax notoginseng* (Gao et al. 2005), and *Echinacea purpurea* (Wu et al. 2007). Although root cultures of *H. perforatum* have also been established by other authors (Cui et al. 2010; Goel et al. 2009), the capacity of these cultures to produce xanthones has never been explored.

As reported by many authors (Pasqua et al. 2003; Zobayed et al. 2004; Franklin and Dias 2006) *H. perforatum* has a high capacity for regeneration, and in our study root regeneration from calli was very easily achieved. The roots were cultivated with IBA, based on our preliminary results and on the results of studies on *H. perforatum* root cultures conducted by Cui et al. (2010) and Goel et al. (2009). According to these studies, IBA and IAA were more effective for lateral root

induction and root growth, whereas NAA was not effective and induced callused roots. Moreover, we chose to use IBA because it is more stable than IAA; in fact, many reports stress that IAA is unstable in culture media at room temperature. Root biomass was positively correlated with the IBA concentration (Fig. 1), though a concentration of 1 mg l^{-1} was the most suitable to obtain morphologically unaltered roots.

The highest concentrations of IBA inhibited xanthone accumulation (Fig. 2), confirming reports that high auxin levels are often deleterious to secondary metabolite accumulation in other species (Dornenburg and Knorr 1995; Chan et al. 2005).

In our study, xanthones were produced in large amounts, with a very stable trend throughout the culture period. To stimulate xanthone accumulation chitosan, a polymer that is able to stimulate the biosynthesis of xanthones in cell cultures of HPA (Tocci et al. 2010) was used. Chitosan acts as an external stimulus on plant cells, which is recognized by receptors localized on the plasma membrane, leading to the induction of a defense response (oxidative burst and phytoalexin production) without penetrating the cell (Kaku et al. 2006). Chitosan treatment resulted in a cessation in growth and a decrease in biomass. Moreover, after elicitation, the xanthone content in roots dramatically increased, peaking after 7 days. This increase supports the hypothesis that xanthones act as a phytoalexin, playing a role in the chemical defense of the plant against fungal pathogens (Franklin et al. 2009). Fifteen days after treatment with chitosan, the xanthone content decreased, and the level at the end of the culture period, day 23, was similar to that of the untreated control. Moreover, after chitosan

treatment, we observed an increased accumulation of these metabolites in the culture medium (Fig. 3c), which constitutes an important advantage for a future scale-up of the production process for the easy recovery of bioactive substances, without destroying plant material.

Our results suggest that root cultures can be considered as a potential tool for large-scale xanthone production, in that accumulation is much higher than that found in the roots of naturally grown plants, and root cultures possess suitable characteristics for a successful scale-up: high biomass growth, stable production of secondary metabolites, sensitivity to external stimuli, and metabolite release in the culture medium.

Regarding the antifungal activity of the extracts, an encouraging level was observed for all of the fungal strains tested: *Candida* spp., the main agent responsible for nosocomial fungal infections (Pfaller and Diekema 2010); *C. neoformans*, a common life-threatening human fungal pathogen (Mitchell and Perfect 1995); and dermatophytes, which are responsible for fungal skin infections (Seebacher et al. 2008). This activity significantly increased when using chitosan, and high activity was found for extracts obtained after 7 days of treatment (mean MIC of 83.4 μg ml^{-1}, 39.1 μg ml^{-1} and 114 μg ml^{-1} against, respectively, *Candida* spp, *C. neoformans*, and dermatophytes). The higher antifungal activity found for extracts from treated roots might be explained by the greater accumulation of xanthones in these roots. Further studies are needed to determine whether the observed activity is the consequence of an increase in xanthones or other unidentified compounds produced in response to elicitation. The increased amount of xanthones and the corresponding improved antifungal activity of the extracts suggest that

xanthones could be the principal constituents responsible for the antifungal activity. Metabolomic analyses are currently being performed to better clarify the extract composition and to perform purification of xanthone-rich fractions to be tested for antifungal activity.

In conclusion, the system described herein is an efficient means of obtaining root biomass which is not subject to pedoclimatic variations or antropic contaminants and which allows extracts with stable quantities of xanthones and good antifungal activity to be obtained. The cytoxicity tests were performed to demonstrate that the root extracts are not toxic for animal cells and to continue studies for an eventual applicative use in human disease. Our preliminary cytotoxicity analyses show a low cytotoxicity of extracts on mammalian cells, though further studies need to be performed.

Acknowledgements. This work was partially supported by the Italian Ministry of Instruction, Universities and Research (Special Project "Fund for Investment on Basic research"- Reti FIRB RBPR05NWWC_006)

References

- Bourgaud F, Gravot A, Milesi S (2001) Production of plant secondary metabolites: a historical perspective. Plant Sci 161: 839-851. doi: 10.1016/S0168-9452(01)00490-3
- Chan LK, Dewi PR, Boey PL (2005) Effect of plant growth regulators on regeneration of plantlets from bud cultures of *Cymbopogon nardus* L. and the detection of essential oils from the *in vitro* plantlets. J Plant Biol 48: 142-145. doi: 10.1007/BF03030574
- Cho JC, Kim JC, Kim MK, Lee HS (2002) Fungicidal activities of 67 herb-derived oils against six phytopathogenic fungi. Agric Chem Biotechnol 45:202-207
- CLSI (2008a) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition. CLSI document M27A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA
- CLSI (2008b) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA, USA
- CLSI (2008c) Reference method for broth dilution antifungal susceptibility testing of yeasts; third informational supplement. CLSI document M27-S3. Clinical and Laboratory Standards Institute, Wayne, PA, USA

- Conceição LFR, Ferreres F, Tavares RM, Dias ACP (2006) Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation. Phytochemistry 67:149-155. doi:10.1016/j.phytochem.2005.10.017
- Conforti F, Statti GA, Tundis R, Bianchi A, Agrimonti C (2005) Comparative chemical composition and variability of biological activity of methanolic extracts from *Hypericum perforatum*. Nat Prod Res 19:295-303. doi: 10.1080/14786410410001715596
- Cui XH, Chakrabarty D, Lee AJ, Paek KY (2010) Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. Bioresour Techonol 101:4708-4716. doi:10.1016/j.biortech.2010.01.115
- Dias ACP, Seabra RM, Andrade PB, Ferreres F, Fernandes-Ferreira M (2000) Xanthone biosynthesis and accumulation in calli and suspended cells of *Hypericum androsaemum*. Plant Sci 150:93-101. doi:10.1016/S0168-9452(99)00178-8
- Dias ACP, Seabra RM, Andrade PB, Ferreres F, Ferreira MF (2000) Xanthone production in calli and suspended cells of *Hypericum perforatum*. J Plant Physiol 158:821-827. doi:10.1078/0176-1617-00195
- Dias ACP, Seabra RM, Andrade PB, Fernandes-Ferreira M (1999) The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from in vivo and in vitro biomass of Hypericum species. J Liq Chromatogr Relat Technol 22:215-227. doi: 10.1081/JLC-100101655

- Dornemburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzym Microb Tech 17:674-684. doi:10.1016/0141-0229(94)00108-4
- Fenner R, Sortino M, Kuze Rates SM, Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nör C, von Poser G, Schapoval E, Zacchino S (2005) Antifungal activity of some Brazilian *Hypericum* species. Phytomedicine 12:236-240. doi:10.1016/j.phymed.2003.11.004
- Ferrari F, Pasqua G, Monacelli B, Cimino P, Botta B (2005) Xanthones from calli of *Hypericum perforatum* subsp. *perforatum*. Nat Prod Res 19:171-176. doi: 10.1080/14786410410001704796
- Finnegan RA, Stephani GM, Ganguli G, Bhattacharya SK (1968) Occurrence of mangiferin in *Hiptage madablota* geartn. J Pharm Sci 57:1039. doi: 10.1002/jps.2600570631
- Fotie J, Bohle DS (2006) Pharmacological and biological activities of xanthones. Anti-Infective Agents Med Chem 5:15-31
- Franklin G, Dias ACP (2006) Organogenesis and embryogenesis in several *Hypericum perforatum* genotypes. In vitro Cell Dev Biol 42:324-330
- Franklin G, Oliveira M, Dias ACP (2007) Production of transgenic *Hypericum perforatum* plants via particle bombardment-mediated transformation of novel organogenic cell suspension

cultures. Plant Sci 6:1193-1203. doi:10.1016/j.plantsci.2007.02.017

- Franklin G, Conceição LFR, Kombrink E, Dias ACP (2009) Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. Phytochemistry 70:60-68. doi:10.1016/j.phytochem.2008.10.016
- Gao X, Zhu C, Jia W, Gao W, Qiu M, Zhang Y, Xiao P (2005) Induction and characterization of adventitious roots directly from the explants of *Panax notoginseng*. Biotechnol Lett 27:1771-1775. doi: 10.1007/s10529-005-3553-4
- Goel MK, Kulreja AK, Bishut NS (2009) *In vitro* manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. Plant Cell Tiss Organ Cult 96: 1-9. doi: 10.1007/s11240-008-9453-2
- Grayer RJ, Harborne JB (1994) A survey of antifungal compounds from higher plants, 1982-1993. Phytochemistry 37:19-42. doi:10.1016/0031-9422(94)85005-4
- Hölzl J, Demisch L, Gollnik B (1989) Investigation about antidepressive and mood-changing effects of *Hypericum perforatum*. Planta Med 55:643
- Hostettmann K, Hostettmann M (1989) Xanthones. In: Dey PM, Harborne JB (eds) Modern methods in plant biochemistry. Academica Press, London, pp 493-507

- Hvoslef-Eide AK, Preil W (2005) Liquid culture systems for in vitro plant propagation. Springer-Verlag, Berlin
- Ishiguro K, Nagareya N, Fukumoto H (1998) A phloroglucinol derivative from cell suspension cultures of *Hypericum patulum*. Phytochemistry 47:1041-1043. doi:10.1016/S0031-9422(98)80068-X
- Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci 103:11086-11091. doi: 10.1073/pnas.0508882103
- Kitanov GM, Blinova KF (1987) Modern state of the chemical study of species of the genus *Hypericum*. Chem Nat Compd 23:151–166. doi:1007/BF00598748
- Kolewe ME, Gaurav V, Roberts SC (2008) Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharmaceut 5:243-256. doi: 10.1021/mp7001494
- Lavault M, Landreau A, Larcher G, Bouchara JP, Pagniez F, Le Pape P, Richomme P (2005) Antileishmania and antifungal activities of xantholides isolated from *Xanthium macrocarpum*. Fitoterapia 76:363-366
- Males Z, Brantner AH, Sovic K, Pilepic KH, Plazibat M (2006) Comparative phytochemical and antimicrobial investigations of

Hypericum perforatum L. subsp. *perforatum* and *Hypericum perforatum* subsp.*angustifolium* (D.C.) Gaudin. Acta Pharm 56:359-367

- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol Methods 65**:**55-63. doi:10.1016/0022-1759(83)90303-4
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x
- Murthy HN, Hahn EJ, Paek KY (2008) Adventitious roots and secondary metabolism. Chin J Biotechnol 24:711–716. doi:10.1016/S1872-2075(08)60035-7
- Nahrstedt A, Butterweck V (1997) Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. Pharmacopsychiatry 30:129–134. doi: 10.1055/s-2007-979533
- Nahrstedt A, Butterweck V (2010) Lessons learned from herbal medicinal products: the example of St. John's Wort (perpendicular). J Nat Prod 28:1015-1021
- Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tiss Organ Cult 81:287–300. doi: 10.1007/s11240-004-6648-z
- Pasqua G, Avato P, Monacelli B, Santamaria AR, Argentieri MP (2003) Metabolites in cell suspension cultures, calli, and in vitro

regenerated organs of *Hypericum perforatum* cv. Topas. Plant Sci 165:977-982. doi:10.1016/S0168-9452(03)00275-9

- Pfaller MA, Diekema DJ (2010) Epidemiology of invasive mycoses in North America. Cri Rev Microbiol 36:1–53. doi: 10.3109/10408410903241444
- Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA, The Global Antifungal Surveillance Group (2010) Results from the ARTEMIS DISK global antifungal surveillance study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J Clin Microbiol 48:1366-77. doi:10.1128/JCM.43.12.5848-5859.2005
- Pinto E, Afonso C, Duarte S, Vale-Silva L, Costa E, Sousa E, Pinto M (2011) Antifungal activity of xanthones: evaluation of their effect on ergosterol biosynthesis by high-performance liquid chromatography. Chem Biol Drug Des. doi: 10.1111/j.1747-0285.2010.01072.x
- Radulovic N, Stankov-Jovanovic V, Stojanovic G, Smelcerovic A, Spiteller M, Asakawa Y (2007) Screening of in vitro antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans. Food Chem 103:15-21. doi:10.1016/j.foodchem.2006.05.062

- Rapp RP (2004) Changing strategies for the management of invasive fungal infections. Pharmacotherapy 24:4–28. doi: 10.1592/phco.24.3.31S.33152
- Rath G, Potterat O, Mavi S, Hostettmann K (1996) Xanthones from *Hypericum roeperanum*. Phytochemistry 2:513-520. doi:10.1016/0031-9422(96)00284-1
- Sakar MK, Tamer AU (1990) Antimicrobial activity of different extracts from some *Hypericum* species. Fitoterapia 61:464-466
- Sakar MK, Tamer AU, Tokur S (1988) Antimicrobial activities of some *Hypericum* species growing in Turkey. Fitoterapia 59:49-52
- Schmidt W, Abd El-Mawla AMA, Wolfender JL, Hostettmann K, Beerhues L (2000) Xanthones in cell cultures of *Hypericum androsaemum*. Planta Med 66:380-381. doi: 10.1055/s-2000-8542
- Schwob I, Bessiere JM, Dhernomez Viano J (2002) Composition and antimicrobial activity of essential oil of *Hypericum coris*. Fitoterapia 73:511-513. doi:10.1016/S0367-326X(02)00171-5 Seebacher C, Bouchara JP, Mignon B (2008) Updates on the epidemiology of dermatophyte infections. Mycopathology 166:335–352. doi: 10.1007/s11046-008-9100-9
- Srivastata S, Srivastata AK (2007) Hairy root culture for mass-production of high-value secondary metabolites. Cri Rev Biotechnol 27:29-43. doi: 10.1080/07388550601173918
- Tocci N, Ferrari F, Santamaria AR, Valletta A, Rovardi I, Pasqua G (2010) Chitosan enhances xanthone production in *Hypericum*

perforatum subsp. *angustifolium* cell cultures. Nat Prod Res 24: 286-293. doi: 10.1080/14786410903006353

- Wang H, Hou AJ, Zhu GF, Chen DF, Sun HD (2005) Cytotoxic and antifungal isoprenylated xanthones and flavonoids from *Cudrania fruticosa*. Planta Med 71:273-274. doi: 10.1055/s-2005-837829
- Wang Y (2008) Needs for new plant-derived pharmaceuticals in the post-genome era: an industrial view in drug research and development. Phytochem Lett 7:395–406. doi: 10.1007/s11101-008-9092-6
- Wu CH, Murthy HN, Hahn EJ, Paek KY (2007) Large-scale cultivation of adventitious roots of *Echinacea purpurea* in airlift bioreactors for the production of cichoric acid, chlorogenic acid and caftaric acid. Biotechnol Lett 29:1179-1182. doi: 10.1007/s10529-007-9399-1
- Ziv M (2005) Simple bioreactors for mass propagation of plants. In: Hvoslef-Eide AK and Preil W, (eds) Liquid culture systems for in vitro plant propagation. Springer-Verlag, Berlin, pp. 79-93
- Zobayed SMA, Murch SJ, Rupasinghe HPV, Saxena PK (2004) *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). Plant Sci 166:333-340. doi:10.1016/j.plantsci.2003.10.005

Annexes to the study not included in the paper

Annex 1

Isolation and chemical characterization of xantones

Materials and methods

Xanthones from roots were isolated applying different methodologies:

- Exclusion size chromatrography on LH-20 resin (Sigma,),using methanol as eluent
- Column chromatography with Silicagel 60 (0.063-0.200 mm) (Merk,), using ethyl acetate/ hexan 1:1 or 1:2 according to the sample characteristics.
- Reverse phase chromatography performed on a LiChroprep RP-18 (40-63 μm) column (Merck chemicals) connected to a Duramat pump working at 70 bar, and a gradient of methanol 10-50 % as eluent.
- Thin Layer Chromatography (TLC) on alumina plates covered with silica gel 60 fluorescent at 254 nm or RP8 (Merck) with various elution systems.
- Preparative TLC on glass plates (20x20 cm) coated with silica gel 60 (250 μm) and fluorescent at 254 nm, using as mobile phase hexan/ ethyl acetate 1:1 or 1:2 according to the characteristics of the sample.

The spots on TLC were visualized by spraying a solution of solphoric acid, phormaldeyde and water on the plates and exposing the plates at high temperatures. The H-NMR spectra were performed in deuterate acetone in a Varian Gemini 200 Spectrometer.

Results

Isolation of 1,7-dihydroxyxanthone

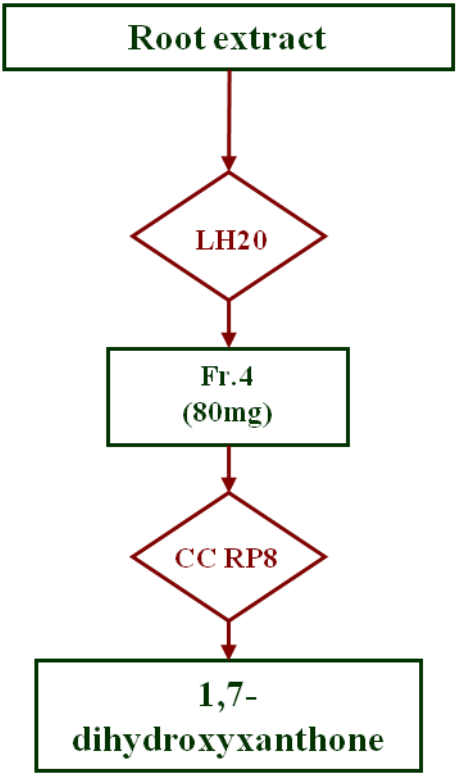


Figure 1 Schematic representation of isolation procedure for 1,7-dihydroxayxanthone. LH20, size exclusion chromatography; CC RP8, reverse phase column chromatography.

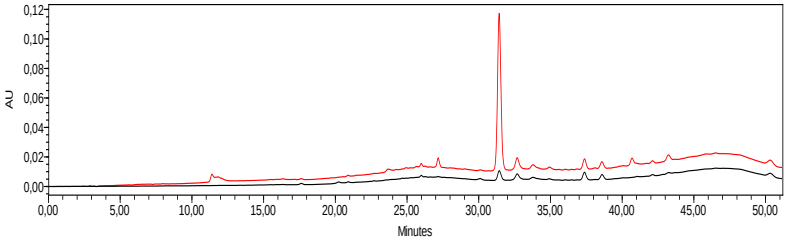


Figure 2 HPLC chromatogram of the isolated 1,7-dihydroxyxanthone.

^{1}H NMR data for 1,7-dihydroxyxanthone in acetone-d_6:

δ: 7.71 (H-3, t, J = 8.0 Hz), 7.62 (H-5, d, J = 3.2 Hz), 7.54 (H-8, d, J= 9.2 Hz), 7.45 (H-7, dd, J= 9.2 e 3.2 Hz), 7.02 (H-4, bd), 6.78 (H-2, bd).

Isolation of 5-O-methyl-2-deprenylrheediaxanthone B

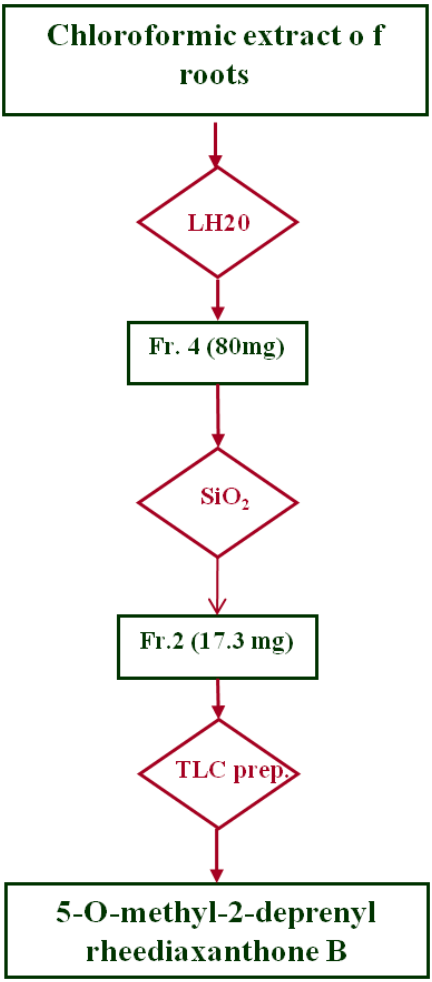


Figure 3 Schematic representation of isolation procedure for 5-O-methyl-2-deprenylrheediaxanthone B. LH20, size exclusion chromatography; SiO2, column chromatography on silica gel; TLC prep., preparative TLC.

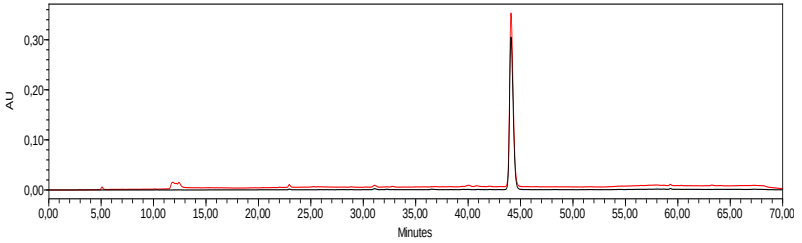


Figure 4 HPLC chromatogram of the isolated 5-O-methyl-2-deprenylrheediaxanthone B.

^{1}H NMR data for 5-O-metil-2-deprenilrheediaxantone B in acetone-d_6

δ: 13.40 (OH-1, s), 7.85 (H-8, d, J = 8.7 Hz), 7.03 (H-7, d, J = 8.7 Hz), 6.18 (H-2, s), 4.60 (H-2', q, J = 6.6 Hz), 4.02 (OMe, s), 1.65 (4'-CH_3 , s), 1.43 (3'-CH_3, d, J= 6.6 Hz), 1.35 (5'- CH_3, s)

Chapter 6:

Establishment of a three-step-culture system and optimization of xanthone production

Root cultures of *Hypericum perforatum* subsp. *angustifolium* revealed to be a potential tool for the production of xanthone-rich extracts. It is known that *in vitro* organ growth and secondary metabolite production are often governed by different combinations of growth regulators and that plant secondary metabolism is generally stimulated when cell growth is slowed. In the light of these assumptions, experiments to test the effect of growth regulators on xanthone production and root growth were performed and a three-step culture system was established. The first step consisted of culturing roots in a medium stimulating organ biomass increase; the second step consisted of subculturing roots in a medium enhancing secondary metabolite production and the third step in the addition of chitosan to further increase total xanthones. The resulting xanthone-rich extract has been tested for antifungal activity to confirm a positive linear correlation between xanthones and antifungal activity.

Chapter 7:

A three-step culture system to increase the xanthone production and antifungal activity of *Hypericum perforatum* subsp. *angustifolium* in vitro roots

Paper published in Plant Physiology and Biochemistry 57 (2012) 54-58

DOI: 10.1016/j.plaphy.2012.04.014

Received 15 March 2012/Accepted 23 April 2012/Available online 8 May 2012

A three-step culture system to increase the xanthone production and antifungal activity of *Hypericum perforatum* subsp. *angustifolium* in vitro roots

Noemi Tocci[a*], Felicia Diodata D'Auria[b*], Giovanna Simonetti[b], Simona Panella[b], Anna Teresa Palamara[b], Gabriella Pasqua[a,**]

[a]Department of Environmental Biology, [b] Department of Public Health and Infectious Diseases, "Sapienza" University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

*Noemi Tocci and Felicia Diodata D'Auria contributed equally to this work.

ABSTRACT

Hypericum perforatum is a well-known medicinal plant. Among all secondary metabolites produced by this species, xanthones are very interesting for their antifungal activity. In the present study, with the aim to improve xanthone production and antifungal activity of *Hypericum perforatum* subsp. *angustifolium* (sin. Fröhlich) Borkh in vitro roots, a new methodology consisting of a three-step culture system, has been

developed. Regenerated roots of *Hypericum perforatum* were cultured in a three-step culture system: in the first step, to increase biomass, the roots were cultured in half-strength liquid Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} indole butyric acid (IBA) and 1.5% sucrose. In the second and third steps, to stimulate secondary metabolism, the roots were cultured with 1.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.215 mg L^{-1} kinetin (KIN), and 0.186 mg L^{-1} 1-naphthalenacetic acid (NAA). In the third step, some of the roots were treated with chitosan. Xanthone production increased 2.7 times following the three-step method. The highest minimal inhibitory concentration (MIC) values were of 36.9, 26.7, and 65 μg mL^{-1}, against *Candida* species, *Cryptococcus neoformans* and dermatophytes, respectively.
A positive correlation between xanthone accumulation and antifungal activity has been shown.

Key words: *Hypericum perforatum,* xanthones, three-step root culture system*, Candida* species*, Cryptococcus neoformans,* dermatophytes

Abbreviations: MS, Murashige and Skoog; IBA, indole butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; NAA, 1-naphthalenacetic acid; MIC, minimal inhibitory concentration

1. Introduction

In the past 30 years there has been a dramatic increase in the incidence of fungal infections [1] and a consequent greater need for effective therapy. However, only a limited range of antifungal agents are available,

and some of the most effective agents are toxic. Furthermore, clinical efficacy may be compromised by intrinsic or acquired drug resistance [2]. The increasing resistance of pathogenic fungi to antifungal compounds and the reduced number of available drugs has led to the search for therapeutic alternatives among natural products. In recent years, many studies have been published on the antifungal activity of plant-derived phenolic compounds, including xanthones [3,4]. Several authors have reported the antifungal activity of a broad range of both synthetic and naturally occurring xanthones with respect to *Candida*, *Cryptococcus*, and dermatophytes. In particular, natural and synthetic oxygenated xanthones have been found to be active mainly on dermatophytes [5]. The xanthone α-mangostin, extracted from the fruit of *Garcinia mangostana*, has been shown to inhibit the growth of *Candida albicans* at 1000 μg mL^{-1}[6]. Toxyloxanthone C, isolated from the roots of *Cudrania fruticosa*, has shown antifungal activity against *Candida albicans*, with minimal inhibitory concentrations of 25 μg mL^{-1} [7]. Xanthones isolated from the roots of *Hypericum roeperanum* have exhibited antifungal activity against *Candida albicans* [8], whereas nothing is known about the activity of extracts from *H. perforatum* roots. However, the content of bioactive compounds extracted from wild plants is not constant and may change according to the developmental stage of the plant, the season, and environmental factors. In vitro cultures are an attractive tool for obtaining extracts rich in bioactive metabolites which are not subject to fluctuation. The potentiality of *Hypericum perforatum* cell cultures to produce xanthones has been already demonstrated by the authors [9].

Moreover, we recently established root cultures of *H. perforatum* subsp. *angustifolium* to obtain extracts with a stable chemical composition [10]. The extracts showed antifungal activity against human pathogenic fungi, and there was a positive linear relationship between antifungal activity and xanthone content in the root extracts. In vitro organ growth and secondary-metabolite production are often governed by different combinations of growth regulators. In light of our previous results, in this study we developed a method to increase the content of bioactive metabolites in the extracts; in particular, we applied a new culture system consisting of three steps which differ in the type and concentration of the growth regulators and by the addition of an elicitor. Herein we report the results of the application of this system in terms of the production of bioactive metabolites and antifungal activity against *Candida* species, *Cryptococcus neoformans* and dermatophytes of *H. perforatum* in vitro roots.

2. Results

To achieve the most suitable culture conditions in terms of biomass and secondary metabolite production, the roots were cultured in a newly developed three-step system. In the first step, the roots, which were cultured in Medium 1, showed a growth index (GI) of 5.4, which corresponded to a 6.4-fold increase in biomass with the respect to the initial inoculum (Fig. 1A). In the second step, although the GI of the roots cultured in Medium 2 (Fig 1A) was significantly lower ($P<0.05$) than the GI of the roots cultured in Medium 1, xanthone production was 3.53 times higher (i.e., 12.84 mg g^{-1} DW vs. 3.64 mg g^{-1} DW) (Fig. 1B). The third step, which included the addition of chitosan as an elicitor, led

to a further increase in total xanthone content (1.4 times higher than that in the untreated roots) ($P < 0.05$) (Fig. 1B). Xanthone production in both chitosan-treated and untreated roots peaked at day 15 of culture and then gradually decreased (Fig. 1B). Chitosan induced an increase for all of the xanthones (Table 1).

The main xanthones accumulated were: 5-methoxy-2-deprenylrheediaxanthone B and paxanthone Table 1 and Fig. 2).

Moreover, the chitosan-treated roots produced 1,7-dihydroxyxanthone, which was not detected in the untreated roots (Table 1). After the addition of chitosan, no negative effects were observed on root vitality, although it led to a lower growth of biomass (Fig. 1A). In Table 2, the antifungal activity of roots transferred to Medium 2 and those maintained in Medium 1 is shown. The mean MIC value for roots cultured in Medium 2, compared to the mean MIC value for roots maintained in Medium, was 10 times lower for *C. albicans*, 5.4 times lower for the non-*albicans Candida* species, 5.9 times lower for *C. neoformans* and 5.2 times lower for dermathopytes. In the third step, the addition of chitosan led to an increase of antifungal activity with the mean MIC values of 36.9 μg mL^{-1} for *Candida* spp., 26.7 μg mL^{-1} for *C. neoformans,* and 65 μg mL^{-1} for dermatophytes (Table 3). Moreover, the root extracts showed a low cytotoxicity for both MDCK and HeLa cells (i.e., CC_{50} >500 μg mL^{-1}).

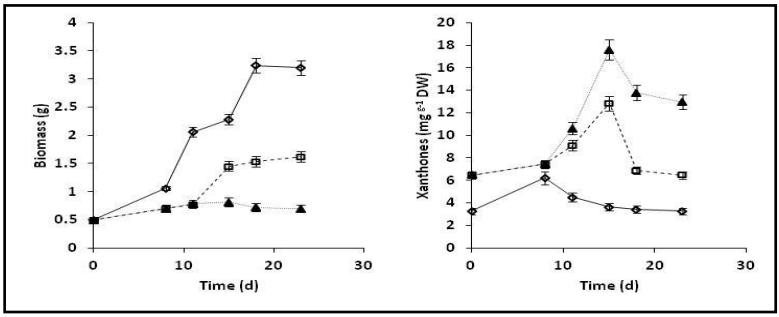


Fig. 1. (A) Growth curves of roots cultured in the Media 1, Medium 2 and Medium 2 supplemented with chitosan. **(B)** Accumulation of xanthones in roots cultured in the Media 1, Medium 2 and Medium 2 supplemented with chitosan during the entire growth period. Results are expressed as means (±SD) of three independent replicates. (◊) Medium 1, (□) Medium 2, ▲ Medium 2 supplemented with chitosan.

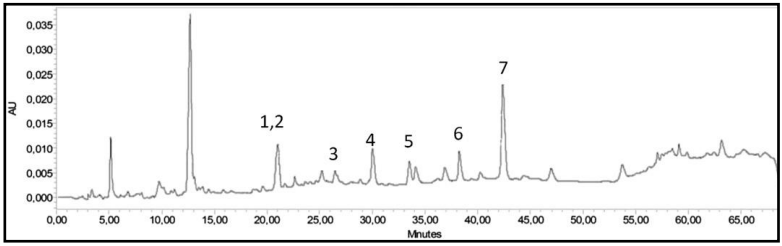


Fig. 2. Chromatographic profile at 260 nm of the extract from roots cultured in Medium 2 supplemented with chitosan, at day 15 of culture. Xanthones: (1, 2) 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, (3) 1,7-dihydroxyxanthone, (4) cadensin G, (5) toxyloxanthone B, (6) paxanthone, (7) 5-O-methyl-2-deprenylrheediaxanthone B.

Table 1 Xanthones detected in the extracts of *Hypericum perforatum* subsp. *angustifolium* roots cultured in Medium 2 untreated (-) and treated with chitosan (+) (third step).

Xanthone[a]	Day 0	Day 8	Day 11		Day 15		Day18		Day 23	
	-	-	-	+	-	+	-	+	-	+
	mg g^{-1} DW[b]	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW	mg g^{-1} DW
1, 2	0.3 ±0.01	0.52 ±0.02	0.47 ±0.02	0.59 ±0.02	nd	1.01 ±0.08	0.45 ±0.02	0.6 ±0.03	0.3 ±0.05	0.67 ±0.02
3	nd	nd	nd	nd	nd	0.99±0.06	nd	1.22±0.06	nd	0.45±0.01
4	0.53 ±0.02	0.62 ±0.02	0.66 ±0.03	0.74 ±0.02	1.38 ±0.07	1.22 ±0.07	0.58 ±0.03	0.98 ±0.09	0.53 ±0.03	0.49 ±0.02
5	0.29 ±0.03	0.43 ±0.01	0.55 ±0.02	0.92 ±0.03	0.66 ±0.03	1.06 ±0.03	0.36 ±0.01	1.34 ±0.12	0.29 ±0.01	1.3 ±0.03
6	1.54 ±0.06	1.51 ±0.08	1.4 ±0.08	1.63 ±0.12	2.37 ±0.09	3.45 ±0.19	1.53 ±0.12	2.87 ±0.15	1.54 ±0.15	2.4 ±0.12
7	3.82 ±0.12	4.45 ±0.07	6.04 ±0.11	6.75 ±0.25	8.43 ±0.23	9.89 ±0.23	3.96 ±0.25	6.76 ±0.35	3.82 ±0.32	7.64 ±0.25
Total	6.48 ±0.24 (a)[c]	7.43 ±0.18 (b)[c]	9.12 ±0.26 (c)[c]	10.63 ±0.54 (d)[c]	12.84 ±0.42 (e)[c]	17.62 ±0.66 (f)[c]	6.88 ±0.43 (a)[c]	13.77 ±0.80 (e)[c]	6.48 ±0.56 (a)[c]	12.95 ±0.45 (e)[c]

[a](1,2) mixture of 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, (3) 1,7-dihydroxyxanthone, (4) cadensin G, (5) toxyloxanthone B, (6) paxanthone, (7) 5-O-methyl-2-deprenylrheediaxanthone B; [b]DW=dry weight; nd= not detected; [c]Different letters refer to significant differences ($P<0.05$).; The data are the means ± standard deviation of three replicates.

Table 2 Comparison of the antifungal activity of extracts of the *Hypericum perforatum* subsp. *angustifolium* roots grown and maintained in Medium 1 and those transferred to Medium 2.

	Roots in Medium 1			Roots in Medium 2			P value[d]
	MIC ($\mu g\ mL^{-1}$)						
Strains[e] (number)	MIC[a]±SD[b]	Range	MIC_{100}[c]	MIC[a]±SD[b]	Range	MIC_{100}[c]	
Candida albicans (3)	192±70.1	128-256	916.7±204.1	18.7±6.5	16-32	74.7±43.7	p<0.001
non-*albicans* *Candida* species (6)	343±270.3	128-1000	805±252	64±39.2	16-128	166.41±44.5	p<0.05
Cryptpoccus neoformans (2)	282±252	64-512	256±0	48±18.5	32-64	128±0	
Dermatophytes (3)	333±172	128-512	656±331	64±0	64	115.2±28.6	p<0.05

3. Discussion

The increasing occurrence of fungal infections is a major public-health concern, and additional antifungal agents must be developed to successfully control the human fungal pathogens that are resistant to available antifungals. Phytomedicine, which has historically been an important aspect of traditional medicine in non-industrialized countries, is now becoming an integral part of healthcare in industrialized nations.

Plants are the source of thousands of new phytochemicals, and different strategies can be applied to improve the yields of bioactive metabolites in the plant and to obtain standardized extracts. We recently evaluated a biotechnological system, in vitro root cultures of *H. perforatum* subsp. *angustifolium* [10], as an effective method for obtaining qualitatively and quantitatively standardized extracts.

Table 3 Antifungal activity, against human fungal pathogens, of total extracts of *Hypericum perforatum* subsp. *angustifolium* roots collected at day 15 of the third step of culture.

	Untreated roots			Chitosan-treated roots			Fluconazole		
	MIC (µg mL^{-1})								
Strains (number)	**MIC[a]±SD[b]**	**Range**	**MIC_{100}[c]**	**MIC[a]±SD[b]**	**Range**	**MIC_{100}[c]**	**MIC[a]±SD[b]**	**Range**	**MIC_{100}[c]**
Candida albicans (16)	61.8±42.3	8-64	129.3±50.2	41.3±27.2	16-64	110.7±57.5	1.5±2.3	0.125-8	88.9±99.1
Candida krusei (4)	42.7±42.2	16-128	128±125	13.3±7.1	8-32	85.3±56.8	8.7±6.7	0.125-16	224±59.2
Candida glabrata (4)	50.7±16.5	32-64	101.3±57.6	28±7.2	16-32	80±28.9	6.2±5	0.5-16	104±74.1
Candida parapsilosis (4)	56.5±66.5	16-256	104.5±95.6	33.1±17.6	16-256	66.1±43.6	1.1±0.8	0.125-2	5.4±4
Candida tropicalis (2)	109.7±71.2	64-256	237.7±136.8	68.6±28.8	32-128	132.6±65.1	1.2±0.2	1-2	128±90.5
Cryptococcus neoformans (12)	37.3±12.3	32-64	106.7±32	26.7±7.8	16-32	96±32.9	1.7±1.2	0.5-4	17.5±19.5
Trichophyton mentagrophytes (6)	74.7±24.9	64-128	96±33.4	41.3±17.4	16-64	74.7±24.9	9.7±5.8	4-16	27.3±19.7
Trichophyton rubrum (2)	64±0	64	128±0	64±0	64	64±0	1±0	1	16±0
Microsporum gypseum (4)	96±35	64-128	96±35	90.7±42.5	32-128	117.3±74.8	21.33±8.26	16-32	80±52.6
Microsporum canis (2)	64±0	64	128±0	64±0	64	128±0	6±2.82	4-8	12±5.6

[a]MIC: arithmetic mean of minimal inhibitory concentration; [b]SD: standard deviation; [c]MIC_{100}: lowest drug concentration that prevented any discernible growth with respect to the control; [d]T test statistical analysis for the evaluation of significance between the two media; a P value of <0.05 was considered significant and a P value of <0.001 was considered highly significant; [e]Strains tested: *Candida albicans* ATCC90028, ATCC90029, ATCC24433. *Candida parapsilosis* ATCC22019, *Candida parapsilosis* DSM11224, *Candida parapsilosis* DSM5784, *Candida krusei* DSM, *Candida tropicalis* DSM 11953, *Candida glabrata* DSM 11226, *Cryptococcus neoformans* DMS 11959, *Cryptococcus neoformans* DSM 6972, *Microsporum canis* DSM 10708, *Trichophyton mentagrophytes* DMS 4870, *Trichophyton rubrum* DMS4167; Data represent the mean of three separate experiments in triplicate.

To improve the efficiency of this system, we performed new experiments, taking into account that in vitro organ growth and secondary metabolite production are often governed by different combinations of growth regulators and that secondary plant metabolism is generally stimulated when cell growth is slowed [11]. These considerations led us to develop a new three-step culture system. The first step consisted of culturing roots in Medium 1, which contained indole butyric acid (IBA) as auxin, in order to obtain a consistent amount of root biomass. The second step consisted of subculturing roots in Medium 2, which contained 1-naphthalenacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, used to increase secondary metabolite production. Previous studies have shown that NAA stimulated xanthone accumulation in cell suspension cultures of *Hypericum perforatum* [12,13]. In *H. perforatum* subsp. *angustifolium* root cultures, the positive effect of the combined growth regulators (Medium 2) on both xanthone production and antifungal activity is reported for the first time in the present study. The results demonstrate that the roots transferred to Medium 2 had 3.53 times higher xanthone production and from 5 to 10 times higher antifungal activity against *Candida* spp., *C. neoformans,* and dermatophytes, with the respect to the roots maintained in Medium 1 (Table 2). Regarding the third step, the addition of chitosan led to a further increase in both total xanthone content and antifungal activity, in particular against *Candida* spp. (Table 3), confirming that chitosan is an effective elicitor in this in vitro system. The decrease in xanthone accumulation, observed after 15 days of culture, in the medium

supplemented with chitosan (Table 1), could be explained as a deterioration of the xanthones previously produced.

The extracts showed low cytotoxicity against mammalian cells. In conclusion, this three-step culture of *Hypericum perforatum* roots could be a good system to obtain extracts for treating human fungal infections.

4. Materials and methods

4.1 Plant material and tissue culture

Regenerated roots of *H. perforatum* subsp. *angustifolium* (sin. Fröhlich) Borkh were cultured in a three-step-system. In the first step, the roots were cultured for 23 days in half-strength liquid MS medium supplemented with 1 mg L^{-1} IBA and 1.5% (w/v) sucrose (herein referred to as "Medium 1."). In the second step, to stimulate secondary metabolite production, the roots were transferred from Medium 1 to half-strength liquid MS medium supplemented with 1.1 mg L^{-1} 2,4-D, 0.215 mg L^{-1} KIN, and 0.186 mg L^{-1} NAA (referred to as "Medium 2") and cultured for 23 days. For both Media 1 and 2, liquid cultures were established by inoculating 5 g fresh weight of the roots, corresponding to 0.5 g dry weight (DW), in 250-mL Erlenmeyer flasks containing 100 mL. The flasks were shaken at 100 rpm at 25±1°C and maintained in the dark.

In the third step, the roots were subcultured again in Medium 2; after 8 days, some of the roots were treated with chitosan (medium molecular weight, Sigma-Aldrich, Italy) at a final concentration of 200 mg L^{-1}. Samples of roots during the third step were harvested by filtration on days 0, 8, 11, 15, 18 and 23 to perform extraction and chemical analysis.

To compare Medium 1 and Medium 2 in terms of antifungal activity, some of the roots were maintained in Medium 1, subculturing them every 23 days.

4.2 Determination of root biomass

For both Medium 1 and Medium 2, root biomass was determined at the end of culture by recording the dry weight (DW). The roots were separated from the medium by passing them through a stainless steel sieve and washed with distilled water to remove residual medium. They were then dried in an oven at 40°C for 72 h, and the DW was measured. The growth index was calculated as follows:

$$\text{Growth index} = \frac{\text{Initial Dry Weight} - \text{Final Dry Weight}}{\text{Initial Dry Weight}}$$

"Initial dry weight" indicates the weight of the roots before extraction, and "final dry weight" indicates the weight of roots after extraction with methanol.

4.3 HPLC analysis

After exsiccation, the roots were grinded and extracted three times with methanol. The obtained extracts were evaporated to dryness in a rotary evaporator at 40 °C and dissolved in methanol to be analyzed as previously described [9] using an HPLC apparatus consisting of a pump (Waters 1525 Binary HPLC Pump), equipped with a UV detector (Waters 2487 Dual λ Detector) and a reverse phase C_{18} column (4.6 mm x 150 mm; 5 μm; Waters). The extract composition was determined by linear gradient elution, using a modified version of the method of Dias et al. [14]. The mobile phase was a gradient prepared from 100:0.1 (v/v)

water–phosphoric acid (Component A) and methanol (Component B) (Carlo Erba, Milan, Italy); the gradient was from 10% to 50% Component B in 13 min, holding for 10 min, and then from 50% to 95% Component B in 42 min. The injection volume was 20 μL; the mobile phase flow rate was 1.0 mL min^{-1}. The compounds were identified at 260 nm and 320 nm by the external standard method, using, as reference, xanthones previously purified and spectroscopically characterized in our laboratory (1,3,6,7-tetrahydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone, toxyloxanthone B, paxanthone, and cadensin G) [15] 1,7-dihydroxyxanthone and 5-methoxy-2-deprenylrheediaxanthone B were purified from in vitro regenerated roots of *H. perforatum* subsp. *angustifolium* [10]. Xanthones were quantified as paxanthone equivalents at 260 nm.

4.4 Organisms

For the antifungal evaluation, strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), the German Collection of Microorganisms (DSMZ, Braunschweig, Germany) and the Pharmaceutical Microbiology Culture Collection (PMC, Department of Public Health and Infectious Diseases, Sapienza, Rome, Italy) were tested. The strains obtained from the ATCC were *Candida albicans* [ATCC90028, ATCC90029, ATCC10261, ATCC10231, ATCC3153, ATCC2091, ATCC76615, ATCC24433 (tested as reference strain)], *Candida parapsilosis* ATCC22019 (tested as quality control strain), and *Trichophyton mentagrophytes* ATCC9972 (tested as reference strain). The strains obtained from the DSMZ were *C. parapsilosis* (DSM11224, DSM5784), *Candida krusei* DSM6128, *Candida tropicalis* DSM11953,

Candida glabrata DSM11226, *Cryptococcus neoformans* (DSM 11959, DSM6972), *T. mentagrophytes* DSM4870, *Trichophyton rubrum* DSM4167, *Microsporum canis* DSM 10708, and *Microsporum gypseum* DSM 3824. The strains obtained from PMC were *C. albicans* (PMC 1011, PMC 1015, PMC 1023, PMC 1071, PMC 1075, PMC 1083, PMC 1088, PMC 1097), *C. parapsilosis* (PMC 0703, PMC 0711), *C. tropicalis* PMC 0908, *C. krusei* (PMC 0613, PMC 0625, PMC 0639), *C. glabrata* (PMC 0805, PMC 0821, PMC 0843), *C. neoformans* (PMC 2103, PMC2107, PMC 2111, PMC 2123, PMC 2136, PMC 2138, PMC 2142, PMC 2157, PMC 2169, PMC 2185), *T. mentagrophytes* (PMC 6503, PMC 6509, PMC 6515, PMC 6527, PMC 6531, PMC 6552), *T. rubrum* PMC 6612, *M. gypseum* (PMC 7303, PMC 7331, PMC 7342), and *M. canis* PMC 7426. All of the strains were stored and grown in accordance with the procedures of the Clinical and Laboratory Standards Institute (CLSI) [16,17].

4.5 Antifungal susceptibility assays

In vitro antifungal susceptibility was evaluated both for the total extracts of the roots grown in Medium 2, collected on day 15 of the third step, treated and untreated with chitosan, and for the extracts obtained from roots maintained in Medium 1, using the CLSI broth microdilution methods [16,17].

The antifungal reference fluconazole (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was also tested. The final concentration ranged from 0.0078 to 250 μg mL^{-1} for fluconazole and from 1 to 1000 μg mL^{1} for total dry root extracts. The extracts and fluconazole were dissolved previously in dimethyl sulfoxide at concentrations 100 times higher than the highest desidered test concentration and successively diluted in test

medium in accordance with the procedures of the CLSI [16]. Microdilution trays containing 100 μL of serial two-fold dilutions of fluconazole or of root extracts in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, U.S.A) were inoculated with an organism suspension adjusted to attain a final inoculum concentration of 1.0 x 10^3 - 1.5 x10^3 cells mL^{-1} for yeasts and 0.4x10^4 - 5x10^4 CFU mL^{-1} for dermatophytes. The panels were incubated at 35°C and observed for the presence of growth at 48 h (*Candida* spp.) and 72 h (*C. neoformans* and dermatophytes).

Quality control was performed by testing the CLSI-recommended strain *C. parapsilosis* ATCC 22019 [18].The minimal inhibitory concentration (MIC) was defined as, for yeasts, the lowest concentration that showed ≥50% growth inhibition compared with the growth control and, for dermatophytes, the lowest concentration that showed ≥80% growth inhibition compared with the control. The MIC_{100} (i.e., 100% growth inhibition) was also evaluated for all of the substances. All of the experiments were performed in triplicate on three consecutive test days.

4.6 Cell viability assay

The cytotoxicity of total extracts used for antifungal assays was evaluated on Madin-Darby canine kidney cells (MDCK) and Human cervix carcinoma cells (HeLa) obtained from the ATCC (Rockville, MD, USA) by MTT assay (Sigma-Aldrich, St. Louis, MO, USA) [18]. The cells (2x10^4 cells $well^{-1}$) were seeded into 96-well plates containing 100μL of RPMI 1640 (Invitrogen, San Diego, CA, USA) without phenol red, supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA, USA), L-glutamine (0.3mg ml^{-1}), penicillin (100U ml^{-1}), and streptomycin

(100μg ml^{-1}) (EuroClone, Celbio, Milan, Italy). The cells were cultured at 37°C in 5% CO_2. Total extracts, with concentrations ranging from 32 to 500 μg mL^{-1}, were added to the wells. Each concentration and control was tested in four replicates with at least five concentrations. The cells were cultured at 37°C in 5% CO_2 for 24 h. MTT solution was added to each well and the plates were incubated for 3-4 h at 37°C in 5 % CO_2. MTT solvent (Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the intracellular crystal. The plates were then incubated at 37°C in 5% CO_2 for 1 h, and the optical density of each well was measured spectrophotometrically at 570 nm. The cytotoxicity of the root extracts was calculated as the percentage reduction in viable cells with respect to the control culture. The 50% cytotoxic concentration (CC_{50}) was evaluated as the drug concentration required to reduce human cell viability by 50% compared to the drug-free control.

4.7 Statistical analysis

The arithmetic mean and standard deviation (SD) were calculated. Data were analyzed by Student's t test for significance. P values of <0.05 were considered as significant; P values of <0.001 were considered as highly significant.

Acknowledgements

This work was partially supported by the Italian Ministry of Instruction, Universities and Research (Special Project "Fund for Investment on Basic Research"- Reti FIRB RBPR05NWWC_006).

References

4. M.A. Pfaller, D.J Diekema, Epidemiology of Invasive Mycoses in North America, Crit. Rev. Microbiol. 36 (2010)1-53.
5. S.R. Norrby, C.E. Nord, R. Finch, Lack of development of new antimicrobial drugs: a potential serious threat to public health, Lancet Infect. Dis. 5 **(**2005) 115-119.
6. J. Fotie, D.S. Bohle, Pharmacological and Biological Activities of Xanthones, Curr. Med. Chem. 5 (2006) 15-31.
7. T. Arif, J.D. Bhosale, N. Kumar, T.K. Mandal, R.S. Bendre, G.S. Lavekar, R. Dabur, Natural products - antifungal agents derived from plants, J. of Asian Nat. Prod. Res. 11 (2009) 621-638.
8. Pinto, C. Afonso, S. Duarte, L. Vale-Silva, E. Costa, E. Sousa, M. Pinto, Antifungal activity of xanthones: evaluation of their effect on ergosterol biosynthesis by high-performance liquid chromatography, Chem. Biol. Drug Des. 77 (2011) 212-222.
9. R. Kaomongkolgit, K. Jamdee, N. Chaisomboon, Antifungal activity of alpha-mangostin against *Candida albicans*, J. Oral Sci. 51 (2009) 401-406.
10. Y. Wang, A. Hou, G. Zhu, D. Chen, H. Sun, Cytotoxic and Antifungal Isoprenylated Xanthones and Flavonoids from *Cudrania fruticosa*, Planta Med. 71 (2005) 273-274.
11. G. Rath, O. Potterat, S. Mavi, K. Hostettmann, Xanthones from *Hypericum roeperanum*, Phytochem. 43 (1996) 513-520.
12. N. Mulinacci , C. Giaccherini, A. R. Santamaria, R. Caniato, F. Ferrari, A. Valletta, F. F. Vincieri, G. Pasqua, Anthocyanins and xanthones in the calli and regenerated shoots of *Hypericum perforatum* var. *angustifolium* (sin. Fro¨hlich) Borkh, Plant Physiol. Biochem. 46 (2008) 414-420.

13. N. Tocci, G. Simonetti, F.D. D'Auria, S. Panella, A.T. Palamara, A. Valletta, G. Pasqua, Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity, Appl. Microbiol. Biotechnol. 91 (2011) 977-987.
14. F. Bourgaud, A. Gravot, S. Milesi, E. Gontier, Production of plant secondary metabolites: a historical perspective, Plant Sci. 161 (2001) 839-851.
15. A.C.P. Dias, R.M. Seabra, P.B. Andrade, F. Ferreres, M.F. Ferreira, Xanthone production in calli and suspended cells of *Hypericum perforatum*, J. Plant Physiol. 158 (2001) 821-827.
16. N.Tocci, F. Ferrari, A.R. Santamaria, A. Valletta, G. Pasqua, Chitosan enhances xanthone production in *Hypericum perforatum* subsp. *angustifolium* cell cultures. Nat. Prod. Res. 24 (2010) 286-93.
17. A.C.P. Dias, R.M. Seabra, P.B. Andrade, M. Fernandes-Ferreira, The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from in vivo and in vitro biomass of *Hypericum* species, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 215-227.
18. F. Ferrari, G. Pasqua, B. Monacelli, P. Cimino, B. Botta, Xanthones from calli of *Hypericum perforatum* subsp. *perforatum,* Nat. Prod. Res. 19 (2005) 171-176.
19. Clinical and Laboratory Standards Institute, Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard M27-A3 (2008) Wayne, PA.

20. Clinical and Laboratory Standards Institute, Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard M38-A2 (2008) Wayne, PA.
21. Clinical and Laboratory Standards Institute, Reference method for broth dilution antifungal susceptibility testing of yeasts; Informational Supplement M 27-S3 (2008) Wayne, PA..
22. T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, J. Immunol. Methods 65 (1983) 55-63.

Chapter 8:

Effect of water-soluble chitosan derivatives on xanthone production in *H. perforatum* subsp. *angustifolium* root cultures

Chitosan proved to be an enhancer of xanthone production in *Hypericum perforatum* root cultures. In spite of the chitosan efficacy, the poor solubility in water of chitosan is the major limiting factor in its utilization in large scale production systems. The semisynthetic carboxymethyl-chitosan is a water soluble chitosan derivative. With the aim to optimize the condtions for the scale up process of root cultures, the effect of carboxymethylchitosan and its derivatives on xanthone production and on the antifungal activity of the root extracts deriving from the treatments has been studied.

Chapter 9

Bioassay-guided fractionation of extracts from *H. perforatum in vitro* roots treated with carboxymethylchitosans and antifungal activity against human fungal pathogens

Paper submitted to Phytomedicine (International Journal of Phytoterapy and Phytofarmacology)

Bioassay-guided fractionation of extracts from *H. perforatum in vitro* roots treated with carboxymethylchitosans and antifungal activity against human fungal pathogens

Noemi Tocci[a§], Felicia Diodata D'Auria[b§] ,Giovanna Simonetti[b], Panella Simona[b], Anna Teresa Palamara[b], Aline Debrassi[c], Clovis Antonio Rodrigues[c], Valdir Cechinel Filho[c], Fabio Sciubba[d], Gabriella Pasqua[a*].

[a]Department of Environmental Biology; [b]Department of Public Health and Infectious Diseases, [d]Department of Chemistry "Sapienza" University of Rome; Piazzale Aldo Moro 5; 00185 Rome, Italy; [c]Curso de Pós-Graduação em Ciências Farmacêuticas, Universidade do Vale do Itajaí (UNIVALI), CEP 88302-202, Itajaí, SC, Brazil.

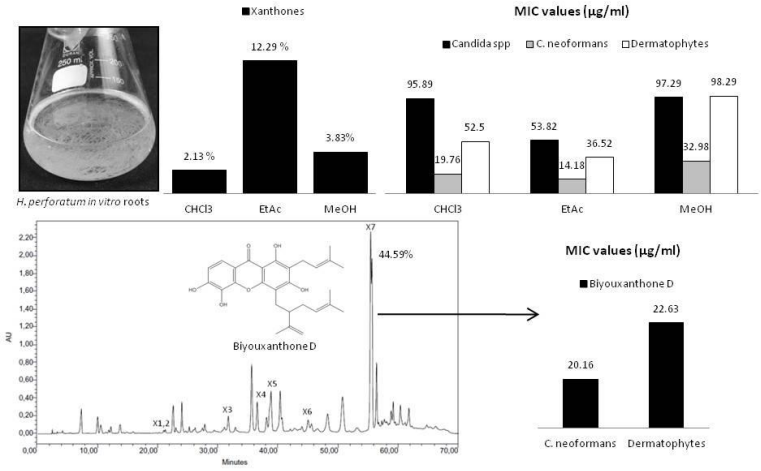
§The authors Noemi Tocci, and Felicia Diodata D'Auria equally contributed to the work.

Abstract

The aim of this study was to individuate, by bioassay-guided fractionation, promising antifungal fractions and/or constituents from *Hypericum perforatum* subps. *angustifolium in vitro* roots. Treatments with O-carboxymethylchitosan (CMC) and its derivatives were used to improve xanthone production in the roots. The bioassay-guided fractionation of CMC-treated roots led to the individuation of a ethylacetate fraction, containing the highest amount of xanthones (6.8%) and showing the

best antifungal activity with MIC values of 53.82, 14.18, and 36.52 μg/ml, against *Candida* spp, *C. neoformans* and dermatophytes, respectively. From this fraction the prenylated xanthone, biyouxanthone D has been isolated and represented the 44.59% of all xanthones detected. For the first time in the present paper biyouxanthone D has been found in *H. perforatum* roots and tested against *C. neoformans*, dermatophytes, and *Candida* species. The xanthones showed the greatest antifungal activity against *C. neoformans* and dermatophytes, with MIC values of 20.16, 22.63 μg/ml. In conclusion, the results obtained in the present study demonstrated that CMC-treated Hpa *in vitro* root extracts represent a tool for the obtainment of promising candidates for further pharmacological and clinical studies.

Graphical abstract



Key words: St John's wort, xanthone, biyouxanthone D, *Cryptococcus neoformans*, dermatophytes, *Candida* species

Introduction

The past few decades have been characterized by a worldwide increase in the incidence of fungal infections (candidiasis and other fungal diseases). The majority of clinically used antifungals show disadvantages such as undesirable side effects or rapid development of resistance and, as a consequence, new antifungal agents are needed to improve the antifungal therapy (Malheiros et al., 2005). This has led to search for therapeutic alternatives among natural compounds. Phytochemical content of wild grown plants is subjected to variation and is influenced by environmental conditions. I*n vitro* cultures, guarantee both the growth of plant biomass under controlled conditions and the obtainment of extracts standardized in chemical composition.

Standardized xanthone-rich extracts from *in vitro* regenerated *Hypericum perforatum* subps. *angustifolium* (Hpa) showed interesting antifungal activity against human pathogen fungi (*Candida* species, *Cryptococcus neoformans*, dermatophytes) (Tocci et al., 2011 and 2012). It was observed that the antifungal activity was positively correlated with the xanthone concentration of the Hpa *in vitro* root extracts. The treatment of roots with chitosan, in fact, stimulated xanthone biosynthesis and in parallel improved the antifungal activity of the extracts (Tocci et al., 2011 and 2012). Chitosan is a linear aminopolysaccharide of glucosamine and *N*-acetylglucosamine units largely used in various industrial countries. Chitosan exerts antimicrobial activity and is able to induce host defense responses in both monocotyledons and dicotyledons. It has been shown

that chitosan induces the production of glucanohydrolases, phenolic compounds and synthesis of specific phytoalexins with antifungal activity (Bautista-Banhos et al., 2006). In spite of the chitosan advantages, the poor solubility, low surface area, and porosity of chitosan are the major limiting factors in its utilization for applicative purposes. Chemical modifications of chitosan are increasingly studied for the potential of providing new applications.

In the present study the effect of O-carboxymethylchitosan (CMC) and its derivatives on xanthone production from Hpa *in vitro* regenerated roots has been evaluated. CMC is a water-soluble chitosan derivative, which has been widely studied because it is easily synthesized, ampholytic character and possibilities of numerous applications and very low toxicity (Mourya et al., 2010; De Oliveira et al., 2012). CMC has been modified by introducing alkyl (hexanoyl anhydride, linoleic acid) or acyl groups (substituted benzaldehydes) in order to increase solubility in water and organic solvents (Yin et al., 2012; Rosa et al., 2011, Wiarachai et al., 2012). Moreover the aim of the present study was to individuate, by bioassay-guided fractionation, fractions and constituents of Hpa *in vitro* root extracts showing antifungal activity against human fungal pathogens.

Materials and Methods

Plant material

In vitro regenerated roots of Hpa were obtained as described by Tocci et al., 2011 and cultured in half strength MS medium (Murashige and Skooge, 1964) supplemented with 1 mg/g indolbutyric acid (IBA) and 1.5% (w/v) sucrose for 23 days according to the growth curve. Liquid

cultures were established by inoculating 5 g fresh weight of the roots, corresponding to 0.5 g dry weight (DW), in 250ml Erlenmeyer flasks containing 100 ml. The flasks were shaken at 100 rpm at 25 °C and maintained in the dark.

Synthesis of O-carboxymethylchitosan

Chitosan (Mw 265 g/mol, deacetylation degree 80%) was obtained from Purifarma, Brazil. All the reagents used were of analytical reagent quality, purchased from Vetec, Brazil. O-carboxymethylchitosan (CMC) was prepared by a modification of the method described by Chen and Park (2003). In brief, 10 g of chitosan were suspended in 100 ml of a solution containing 13.5 g of sodium hydroxide, 80 ml of water, and 20 ml of 2-propanol, at 5 ^{o}C and stirred for 2 h. Monochloroacetic acid (15 g) was dissolved in 2-propanol (25 ml) and added dropwise to the alkalized chitosan. The reaction was stopped by adding 70% (v/v) ethyl alcohol (200 ml) and led to the obtainment of O-carboxymethylchitosan with a degree of carboxymethylation of 44.3% (determined by conductometric titration).

Synthesis of O-carboxymethylchitosan amphiphilic derivatives

OCCh (5 g) was dispersed into a solution made of 300 ml water and one equivalent of aldehyde (benzaldehyde-, 4-methoxybenzaldehyde, 4-hydroxybenzaldehyde and 4-N,N-dimethylaminobenzaldehyde) and methanol at pH 4. After 1 day stirring, $NaBH_4$ was added and the mixture was stirred for 1.5 hour. The derivatives were precipitated by adding acetone and dried in vacuum for 24 h as described by Rabea et al., 2005.

The ^{1}H NMR analysis was performed using a Bruker Nuclear Magnetic Resonance 300 MHz Spectrometer and the polymers were dispersed in CD_3COOD and D_2O.

The reactions between O-CMCh and benzaldehyde, 4-methoxybenzaldehyde, 4-hydroxybenzaldehyde and 4-N,N-dimethylaminobenzaldehyde, resulted in the following derivatives: N-benzyl-O-carboxymethylchitosan (BzCMC), N-(4-methoxybenzyl)-O-carboxymethylchitosan (N,O-CMC), N-(4-hydroxybenzyl)-O-carboxymethylchitosan (OHCMC), N-(4-N,N-dimethylaminobenzyl)-O-carboxymethylchitosan (N,N-CMC).

Elicitation treatments

The elicitation treatments with different chitosans, were performed according to the protocol previously established and optimized by the authors (Tocci et al., 2011). The elicitors were added at the 8th day of culture at the final concentration of 200 mg/l. Samples of roots were harvested after 8 days of treatment by filtration and subjected to extraction and chemical analysis.

Root extraction and HPLC analysis

After drying, the roots were grinded and subjected to extraction. All the solvents used were purchased by Carlo Erba Reagents, Italy. Samples of roots treated and untreated were extracted three times with methanol. Roots treated with CMC were subjected also to serial extractions with solvent of increasing polarity. The first extraction was performed with chloroform, then roots were left to dry, in order to eliminate any trace of chloroform, and extracted with ethyl acetate and finally with methanol. The obtained extracts were evaporated to dryness in a rotary evaporator

at 40 °C. The extracts were dissolved in methanol (HPLC grade, Carlo Erba Reagents, Italy) and filtered with syringe filter, pore sizes 0.45μm, to remove insoluble particles which could negatively affect the chromatographic separation performance. HPLC analysis were carried out as described by Tocci et al. 2010, using the elution method reported by Dias et al. 1999 with little modifications. Briefly the mobile phase was a gradient prepared from 100:0.1 (*v*/*v*) water–phosphoric acid (component A) and methanol (component B); the gradient program was from 10% to 50% B in 13 min, holding for 10 min, and then from 50% to 95% B in 42 min. The injection volume was 20 μl, the mobile phase flow rate was 1.0 ml/min. The compounds were identified at 260 nm and 320 nm by the external standard method, using, as reference, xanthones that had been previously purified and spectroscopically characterized in our laboratory (1,3,6,7-tetrahydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone, toxyloxanthone B, paxanthone, and cadensin G). 1,7-dihydroxyxanthone and 5-methoxy-2-deprenylrheediaxanthone B were purified from *in vitro* regenerated roots of HPA (Tocci et al., 2011). Xanthones were quantified as paxanthone equivalents at 260 nm.

Isolation of biyouxanthone D

Biyouxanthone D was isolated by preparative thin layer chromatophy (silica gel, hexane/ethyl acetate 1:1) (Carlo Erba, Italy) from the ethylacetate extract of CMC-treated roots. The identification was carried out by nuclear magnetic resonance (NMR). Spectroscopic data were recorded at room temperature on an Avance 400 MHz NMR spectrometer. The identification was carried out by nuclear magnetic resonance (NMR) spectroscopy. Monodimensional ^{1}H and ^{13}C

experiments as well as bidimensional homonuclear ^{1}H-^{1}H TOCSY and bidimensional heteronuclear ^{1}H-^{13}C HSQC experiments were recorded at a temperature of 298 K on an Avance 400 MHz NMR spectrometer employing the pulse sequences present in the spectrometer routines. The univocal resonance was assigned on the basis of TOCSY and HSQC scalar couplings while the identification of quaternary carbons was obtained comparing the ^{13}C NMR chemical shifts (δ) with those of literature data and the results are reported in Table 1.

Organisms

For the antifungal evaluation, strains obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), the German Collection of Microorganisms (DSMZ, Braunschweig, Germany) and the Pharmaceutical Microbiology Culture Collection (PMC, Department of Public Health and Infectious Diseases, "Sapienza" University, Rome, Italy) were tested. The strains were: *Candida albicans* (ATCC 10231, ATCC 10261, ATCC 24433, ATCC 90028, ATCC 90029, PMC 1002, PMC 1016, and 1093), *C. parapsilosis* ATCC22019, *C. parapsilosis* PMC 0703, *C. tropicalis* DSM 11953, *C. tropicalis* PMC 0908, *C. glabrata* PMC 0805, *C. glabrata* PMC 0823, *C. krusei* DSM 6128, and *C. krusei* PMC 0625), *Cryptococcus neoformans* (DSM 11959, PMC 2136, PMC 2123, PMC 2115, PMC 2103, PMC 2107, PMC 2102, and PMC 2111), dermatophytes (*Trichophyton mentagrophytes* DSM 4870, *T. mentagrophytes* PMC 6503, *T. mentagrophytes* PMC 6509, *T. mentagrophytes* PMC 6552, *Trichophyton rubrum* PMC 6612, *Microsporum gypseum* DSM 3824, and *M. gypseum* PMC 7331). All of the strains were stored and grown in

accordance with the procedures of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008a, b).

Antifungal susceptibility assays

In vitro antifungal susceptibility was evaluated for the extracts from root cultures and isolated biyouxanthone D, using the CLSI broth microdilution methods (CLSI,2008a, b). Fluconazole (FLC) was used as reference drug. The final concentration ranged from 1 to 512 μg/ml for total dry root extracts and from 0.125 to 64 μg/ml for Biyouxanthone D and for FLC. The extracts were dissolved previously in dimethyl sulfoxide at concentrations 100 times higher than the highest desired test concentration and successively diluted in test medium in accordance with the procedures of the CLSI (CLSI, 2008c). Microdilution trays containing 100 μl of serial two-fold dilutions of root extracts in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Missouri, U.S.A) were inoculated with an organism suspension adjusted to attain a final inoculum concentration of 1.0×10^3 - 1.5×10^3 cells/ml for yeasts and 0.4×10^4 - 5×10^4 CFU/ml for dermatophytes. The panels were incubated at 35°C and observed for the presence of growth at 48 h (*Candida* spp.) and 72 h (*C. neoformans* and dermatophytes).

The minimal inhibitory concentration (MIC) defined as, for yeasts, the lowest concentration that showed ≥50% growth inhibition compared with the growth control and, for dermatophytes, the lowest concentration that showed ≥80% growth inhibition compared with the growth control was evaluated for all extracts and biyouxanthone D. The results were expressed as the median of the MIC values of five

replicates, and the geometric mean (GM) and the range of the MIC values.

Results

1H NMR analysis of CMC and its derivatives

The 1H NMR spectra of the derivatives showed the characteristic O-CMCh signal, a signal at approximately 7.0 ppm indicating the presence of an aromatic group (benzene ring), and the following peaks: singlet at 3.7 ppm (methoxy group protons) for 4-CH3O-BzCMCh, singlet at 7.1 ppm (phenolic hydroxyl group) for 4-OH-BzCMCh, and singlet at 3.1 ppm (two methyl groups binded to nitrogen on the benzene ring) for 4-N,N-(CH3)2BzCMCh respectively. The SD of the derivatives varies according to the aldehyde and the methodology employed. The SD values were 12 %, 3 %, 27% and 65 %, for BzCMCh, 4-CH3O-BzCMCh, 4-OH-BzCMCh, 4-N,N-(CH3)2BzCMCh respectively.

Bioassay-guided isolation and identification of antifungal compounds

The antifungal activity of total root extracts from root cultures treated with BzCMC and CMC, which at a first analytical screening showed to be the richest in xanthones (Table 1), has been evaluated against *Candida* species, *C. neoformans*, and dermatophytes.

The extract from roots treated with BzCMC showed the geometric mean MIC values of 89.21 μg/ml for *Candida* species (Table 2), 49.15 μg/ml for dermatophytes (Table 3) and 27.52 μg/ml for *C. neoformans* (Table 4). In line with the higher amount of xanthones in the extract from root cultures treated with CMC, an improved antifungal activity has been found. This extract in fact showed an increase of antifungal activity

against all tested fungi, with MIC values of 40.90, 33.07, and 13.76 μg/ml, for *Candida* species, dermatophytes and *C. neoformans,* respectively.

Table 1: Xanthones in *H. perforatum* subsp. *angustifolium* roots after treatment with CMC and its derivatives.

Xanthones	Control	Chitosan	CMC	BzCMC	OHCMC	N,N-CMC
	(mg/g DW)					
1,3,5,6-; 1,3,6,7-tetrahydroxyxanthone	0.37±0.01	1.2±0.04	5.7±0.13	2.8±0.21	0.54±0.02	0.18±0.02
1,7-dihydroxyxanthone	1.66±0.04	8.36±0.39	4±0.36	0.8±0.05	2.23±0.14	0.45±0.01
Toxyloxanthone B	0.35±0.01	2.48±0.12	3.8±0.26	1.99±0.07	0.18±0.02	nd
Paxanthone	0.53±0.02	1.44±0.05	11.92±0.59	2.71±0.12	1.3±0.15	0.61±0.03
5-O-methyl-2-deprenylrheediaxanthone B	0.73±0.03	0.78±0.02	0.98±0.05	0.78±0.04	0.75±0.05	1.72±0.14
Biyouxanthone D	0.25±0.01	2.23±0.15	16.02±0.68	4.48±0.16	3.29±0.15	4.5±0.52
Total	3.89±0.21	16.49±0.77	42.42±2.07	13.56±0.65	8.29±1.18	7.46±0.72

CMC-treated roots were therefore subjected to bioassay-guided fractionation to isolate and identify antifungal constituents (Table 5).

The antifungal activity of three fractions obtained by extraction with chloroform, ethyl acetate and methanol has been evaluated. The ethylacetate fraction showed the greatest activity against *Candida* species (Table 2), dermatophytes (Table 3) and *C. neoformans* (Table 4) among the other tested ones, with MIC values of 53.82, 36.52, and 14.18 μg/ml, respectively. The HPLC qualitative analysis of the ethylacetate fraction

showed the presence of one unidentified major peak with retention time of 56 minutes, among the others obtained from CMC-treated Hpa root.

Table 2: Antifungal activity against *Candida* species of root extracts

	CMC	BzCMC	EtAc	CHCl3	MeOH	Fluconazole
Candida species	MIC(μg/ml)					
C. albicans PMC1002	64	128	64	64	64	0,25
C.albicans ATCC 3153	32	128	32	64	64	16
C.albicans ATCC 10231	64	128	64	128	128	2
C.albicans ATCC 10261	32	64	64	128	64	4
C.albicans ATCC 90028	32	64	32	64	128	0,50
C.albicans ATCC 24433	64	128	64	64	64	1
C.albicans ATCC 90029	32	128	64	128	64	0,25
C.albicans PMC 1016	64	64	128	128	64	0,25
C.parapsilosis DSM 5784	32	128	128	128	128	1
C.parapsilosis PMC 0703	64	128	128	128	128	0,25
C.tropicalis DSM11953	64	128	64	128	128	1
C.tropicalis PMC 0908	64	128	32	64	128	1
C.glabrata PMC 0805	32	64	64	128	64	1
C.glabrata PMC 0823	32	64	64	128	64	1
C.krusei DSM 6128	32	64	16	32	64	16
C.krusei PMC 0625	16	32	16	64	128	16
GM	40.90	89.21	53.82	95.89	97.29	1.23
RANGE	16-64	32-128	16-128	32-128	64-128	0.25-32

MIC: the lowest concentration that showed ≥50% growth inhibition compared with the growth control GM: Geometric mean of the minimal inhibitory.The MIC results were expressed as median

The unkown compound was isolated and purificated by preparative TLC. The process led to the obtainment of 7 mg of compound which

was characterized by ^{1}HNMR (Table 6) and identified as biyouxanthone D by comparison with literature data (Tanaka et al., 2010). Biyouxanthone D was quantified in all the studied extracts and revealed to be the major xanthone (tables 1 and 5). In particular the amount of biyouxanthone D detected in the total extract was very similar to the one detected in the ethylacetate fraction (Table 2). Biyouxanthone D, showed high antifungal activity against dermatophytes and *C. neoformans* with MIC values of 22.63 and 20.16, while higher MIC values were observed for *Candida* spp (data not shown).

Table 3: Antifungal activity against dermatophytes of root extracts obtained from CMC-treated Hpa roots

Dermatophytes	MIC(µg/ml)					
	CMC	BzCMC	EtAc	CHCl3	MeOH	Fluconazole
Trichophyton mentagrophytes DSM 4870	64	64	64	64	64	16
Trichophyton mentagrophytes PMC 6509	32	64	32	64	128	1
Trichophyton mentagrophytes PMC 6552	32	32	32	64	128	16
Trichophyton mentagrophytes PMC 6503	32	64	64	64	128	2
Microsporum gypseum PMC 7303	64	64	32	32	64	16
Trichophyton rubrum PMC 6612	32	64	32	64	64	2
Microsporum gypseum DSM 3824	16	16	32	32	64	16
GM	33.07	49.15	36.52	52.50	98.29	5.56
range	16-64	16-128	16-128	16-128	32-512	1-16

MIC: the lowest concentration that showed ≥80% growth inhibition compared with the growth control.GM: Geometric mean of the minimal inhibitory.The MIC results were expressed as median.

Table 4: Antifungal activity against *Cryptococcus neoformans* of root extracts obtained from CMC-treated Hpa roots

Cryptococcus neoformans	MIC(μg/ml)					
	CMC	BzCMC	EtAc	CHCl3	MeOH	Fluconazole
PMC 2111	32	64	16	16	32	1
PMC 2115	32	64	32	32	32	4
PMC 2102	32	32	16	32	64	4
PMC 2103	16	8	16	16	32	2
PMC 2107	16	64	16	32	64	1
DSM 11959	8	16	8	8	16	2
PMC 2123	16	32	16	16	32	2
PMC 2136	4	32	8	16	16	2
GM	13.76	27.52	14.18	19.76	32.98	2.06
RANGE	4-32	8-64	8-32	8-64	16-64	1-4

MIC: the lowest concentration that showed ≥50% growth inhibition compared with the growth control.GM: Geometric mean of the minimal inhibitory. The MIC results were expressed as median.

Xanthone production in roots after treatments

In all of the analyzed treated samples an increased amount of xanthones was detected (table 1). The composition of the extracts was qualitatively similar but characterized by different concentration of the each of the xanthones. CMC was found to be the best elicitor for xanthones, inducing 10.90 folds the amount detected in the control (42.42 and 3.89 mg/g DW respectively), followed by BzCMC (3.48 folds), OHCMC (2.13 folds) and N,NCMC (1.91 folds). The composition of the extracts differed in the concentration of the xanthones. In a previous work we

found that in chitosan-treated roots, 1,7-dihydroxyxanthone was the main compound accumulated, followed by the toxyloxanthone B (table 1).

Table 5: Xanthones from CMC-treated roots obtained with extraction with different solvents

Xanthones	Total extract		CHCl3		EtAc		MeOH	
	%	mg/g DW	%	mg/g DW	%	mg/g DW	%	mg/g DW
1,3,5,6-; 1,3,6,7-Tetraoxyxanthone	1.8	5.7	Nd	Nd	0.6	0.37	0.69	2.2
1,7-dihydroxyxanthone	1.2	4	0.07	0.051	2.53	1.55	1.37	4.36
Toxyloxanthone B	1.2	3.8	0.19	0.15	1.03	0.63	0.3	0.96
Paxanthone	3.77	11.92	0.4	0.3	1.6	1	0.6	1.9
5-O-methyl-2-deprenylrheediaxanthone B	0.31	0.98	0.46	0.35	1.05	0.66	0.29	0.91
Biyouxanthone D	5.07	16.02	1.01	0.77	5.48	3.45	0.58	1.82
Total	13.35	42.42	2.13	1.62	12.29	7.66	3.83	12.16

The roots treated with CMC and its derivatives accumulated biyouxanthone D as the major xanthone. In particular the CMC treatment induced the highest production of biyouxanthone D (16.02 mg/g DW) among the analyzed samples. CMC- and BzCMC-treated roots accumulated paxanthone as the second major compound (11.92±1.02 and 2.71±0.06 mg/g DW respectively), while in OHCMC- and N,NCMC-treated roots accumulated 1,7-dihydroxyxanthone (2.23±0.04 mg/g DW) and 5-O-methyl-2-deprenylrheediaxanthone B (1.72±0.06 mg/g DW) were the second most produced compounds.

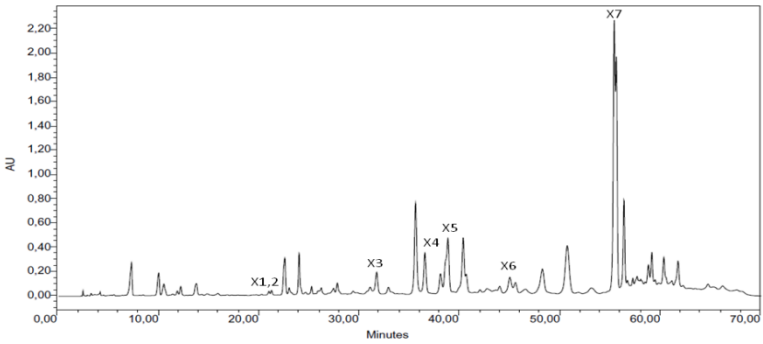


Figure 1: HPLC chromatographic profile at 260 nm of the ethyl acetate extract of Hpa roots treated with CMC. Xanthones: (X1, X2) 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, (X3) 1,7-dihydroxyxanthone, (X4) toxyloxanthone B, (X5) paxanthone, (X6) 5-O-methyl-2-deprenylrheediaxanthone B, (X7) biyouxanthone D.

The extraction of CMC-treated with solvent of increasing polarity led to the obtainment of fractions different in composition (table 5). The fraction deriving from chloroform extraction led to an extract composed for 2.13% of xanthones, of which 1.01% represented by biyouxanthone D and 0.46% by 5-O-methyl-2-deprenylrheediaxanthone B, both the xanthones with less polarity among the detected ones.

The extract obtained with ethylacetate was the richest in xanthones (12.28%) with a value very similar to the one calculated for total extract (13.35%) and was characterized by 5.48% biyouxanthone D and 1.6% paxanthone, followed by 1.05% 5-O-methyl-2-deprenylrheediaxanthone B and 1.03% toxyloxanthone B. The last fraction was obtained through extraction with methanol and was characterized by 3.83% xanthones, with 1.37% 1,7-dihydroxyxanthone and 0.58% biyouxanthone D.

Table 6: ^{1}H and ^{13}C NMR resonance assignment of biyouxanthone D

Position	δ ^{1}H	multiplicity* (J Hz)	Integral	δ ^{13}C
7	6.91	d (8.9)	1	112.46
8	7.69	d (8.9)	1	118.04
4'	1.72	s	3	25.70
5'	1.78	s	3	17.60
1'	3.4	d (6.9)	2	21.36
2'	5.23	m	1	121.00
6"	1.54	s	3	17.60
10" + 7"	1.68	s	3 + 3	19.6 + 25.6
3"	2.12	m	2	31.67
2"	2.32	m (6.9)	1	47.57
1"	2.76	dd (6.9; 14.6)	1	26.95
1"	2.83	dd (6.9; 14.6)	1	26.95
9"	4.62	m	1	110.73
9"	4.68	m	1	110.73
4"	5.17	m	1	122.34
1	\	\	\	158.39
2	\	\	\	108.59
3	\	\	\	160.51
4	\	\	\	105.81
4a	\	\	\	152.69
5	\	\	\	130.21
6	\	\	\	149.00
8a	\	\	\	114.00
9a	\	\	\	102.62
9	\	\	\	180.51
10a	\	\	\	144.68
3'	\	\	\	136.39
5"	\	\	\	133.79
8"	\	\	\	150.00

*s: singlet; d: doublet; dd: double doublet; m: multiplet;

Root cultures are an attractive biotechnological tool for the production of standardized extracts, and for the isolation of bioactive fractions and compounds (Pasqua et al 2005). The large-scale cultivation of untransformed roots for producing important phytochemicals has been achieved for medicinal plants such as *Panax notoginseng* (Gao et al. 2005), and *Echinacea purpurea* (Wu et al. 2007). In our previous works (Tocci et al., 2011 and 2012), we demonstrated that *in vitro* regenerated roots of *H. perforatum* produce higher xanthone amounts if compared with roots from wild grown plants and from *in vitro* grown plantlets (Tocci et al., under revision). Moreover, we proved a positive correlation between xanthone concentration in the extract and antifungal activity against human pathogen fungi. Previous experiments demonstrated that xanthone biosynthesis is sensitive to external stimuli. In particular chitosan and variation in growth regulators combination in the culture medium (Tocci et al., 2012) were able to influence the production of these compounds. This is not surprising in the light of the suggested role of xanthones in the plant. Franklin et al. (2009), in fact, in a study on phenolic metabolism in *H. perforatum* cell cultures, after infection with *Agrobacterium tumefaciens,* showed that xanthones act as phytoalexins and are produced as defense response to pathogen attack. Chitosan and its derivatives are known to form a semi-permeable film around plant tissues, they are inhibitory to a number of pathogenic fungi, and they also induce host-defense responses (El Ghaouth et al. 1994). CMC has shown to enhance chilling tolerance in cucumber seedlings (Sun et al., 2004) and storage protein accumulation in maize seeds (Osuji et al., 1992) and to delay powdery mildew disease in strawberry (Lowe et al.,

2012). To our knowledge this is the first time that the CMC and its derivatives are used as elicitors of bioactive metabolites in *H. perforatum*. In all the analyzed treated samples it has been found that CMC derivatives stimulated the synthesis of xanthones (table 2). In comparison with the others, CMC- and BzCMC-treated roots were characterized by the highest amount of xanthones (42.42 ±2.13 and 13.56±0.16 mg/g DW respectively) and among them the best antifungal activity was found for CMC-treated root extracts against all of the tested strains. The bioassay-guided fractionation of CMC-treated roots led to the individuation of a fraction, obtained by extraction with ethylacetate, containing the highest amount of xanthones (6.8%) and showing the best antifungal activity with MIC values of 53.82, 14.18, and 36.52, against *Candida* spp, *C. neoformans* and dermatophytes, respectively. Our results confirm the positive correlation between xanthone content in the extracts and antifungal activity as already observed in our previous studies (Tocci et al 2011 and 2012).

The analysis of ethylacetate CMC-treated root extracts led to purification of a prenylated xanthone biyouxanthone D, isolated by Tanaka et al. (2010) from *Hypericum chinense* and for the first time in the present paper in *H. perforatum* roots. Biyouxanthone D represented the 44,59 % of all xanthones detected in ethylacetate extract (table 3). The compound, tested for the first time in the present work against *C. neoformans* and dermatophytes showed interesting antifungal activity. Comparing the amount of biyouxanthone D in the tested extracts with the antifungal activity exerted, a linear positive correlation is evident, suggesting that

biyouxanthone D is an active constituent of the *H. perforatum* regenerated roots, against dermatophytes and *C. neoformans*.

Little information is available on mechanism of action of xanthones as antifungals, recently Pinto et al (2011) reported that oxygenated xanthones reduced the biosynthesis of ergosterol. Moreover, it is important to underline that biyouxanthone D is a prenylated compound. It is known that the presence of isoprenoid chains is a major determinant of the bioactivity which resides in a enhanced interaction with biological membranes and in an increased affinity for target proteins (Botta et al., 2006). In conclusion, the results obtained in the present study demonstrated that CMC-treated Hpa *in vitro* root extracts represent a tool for the obtainment of promising candidates for further pharmacological and clinical studies in the developing formulations of new natural antifungal agents.

Acknowledgements

This work was supported by Grant C26A11XLZ5 of the University "Sapienza" Rome, Italy, and by the Project PON 01_01802.

References

- Bautista-Banhos, S., Hernandez-Lauzardo, A.N., Velazquez-del Valle, M.G., Hernandez-Lopeza, M., Ait Barkab, E., Bosquez-Molina, E., Wilson, C.L., 2006. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Protection 25, 108-118.

- Chen, X., Park, H., 2003. Chemical characteristics of *O*-carboxymethyl chitosans related to the preparation conditions. Carbohyd. Polym. 53, 355-359.
- CLSI 2008b. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA, USA.
- CLSI 2008c. Reference method for broth dilution antifungal susceptibility testing of yeasts; third informational supplement. CLSI document M27-S3. Clinical and Laboratory Standards Institute, Wayne, PA, USA.
- CLSI. 2008a. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition. CLSI document M27A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA.
- de Oliveira Rosa, T.R., Debrassi, A., da Silva, R.M.L., Bressan, C., de Freitas, R.A., Rodrigues, C.V., 2011. Synthesis of *N*-benzyl-O-carboxymethylchitosan and application in the solubilization enhancement of a poorly water-soluble drug (triamcinolone). J. Appl. Pol. Sci. 124, 4206–4212.
- Franklin, G., Conceição, L.F.R., Kombrink, E., Dias, A.C.P., 2009. Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. Phytochem. 70, 60-68.

- Gao, X., Zhu, C., Jia, W., Gao, W., Qiu, M., Zhang, Y., Xia, P., 2005. Induction and characterization of adventitious roots directly from the explants of *Panax notoginseng*. Biotechnol. Lett. 27, 1771-1775.
- Liu, T.Y., Chen, S.Y., Lim, Y.L., Liu, D.M., 2006. Synthesis and characterization of amphiphatic carboxymethyl-hexanoyl chitosan hydrogel: water-retention ability and drug encapsulation. Langmuir 22, 9740-9745.
- Lowe, A., Rafferty-McArdle, S.M., Cassells, A.C., 2012. Effects of AMF- and PGPR-root inoculation and a foliar chitosan spray in single and combined treatments on powdery mildew disease in strawberry. Agr. Food. Sci. 21, 28-38.
- Mouryaa, V.K., Inamdara, N.N., Tiwari, A., 2010. Carboxymethyl chitosan and its applications. Adv. Mat. Lett., 1, 11-33.
- Osuji, G.O., Cuer, R.G., 1992. N-Carboxymethylchitosan enhancement of the storage protein contents of maize seeds (Zea mays L.). Food. Biotech. 6, 105-126.
- Pasqua, G., Monacelli, B., Valletta, A., Santamaria, A.R., Fiorillo, F., 2005. Synthesis and/or accumulation of bioactive molecules in the *in vivo* and *in vitro* root. Plant Biosyst. 139, 180-188.
- Rabea, E.I., Badawy, M.E., Rogge, T.M., Stevens, C.V., Hofte, M., Steurbaute, W., Smagghe, G., 2005. Insecticidal and

fungicidal activity of new synthesized chitosan derivatives. Pest Managem. Sc. 6, 951-960.

- Sun, Q., Yu, X., Gao, J., Chang, S., 2004. Effect of Carboxymethyl Chitosan on Chilling Tolerance in Cucumber Seedlings. Sci. Agric. Sinica, DOI: CNKI:SUN:ZNYK.0.2004-11-00C.
- Tanaka, N., Mamemura, T., Abe, S., Imabayashi, K., Kashiwada, Y., Takaishi, Y., Suzuki, T., Takebe, Y., Kubota, T., Kobayashi J., 2010. Biyouxanthones AD, Prenylated Xanthones from Roots of *Hypericum chinense*. Heterocycles 80, 289-302.
- Tocci, N., D'Auria, F.D., Simonetti, G., Panella, S., Palamar, A.T., Pasqua, G. A three-step culture system to increase the xanthone production and antifungal activity of *Hypericum perforatum* subsp. *angustifolium in vitro* roots. Plant Physiol. Biochem. 57, 54-58.
- Tocci, N., Simonetti, G., D'Auria, F.D., Panella, S., Palamara, A.T., Valletta, A., Pasqua, G., 2011. Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity. Appl. Microbiol. Biotechnol. 91, 977-987.
- Wiarachai, O., Thongchul, N., Kiatkamjornwong, S., Hovene, V.P., 2012. Surface quaternized chitosan particles as an alternative and effective organic antibacterial material. Colloid. Surf. B-Biointerfaces 92, 121-129.

- Wu, C.H., Murthy, H.N., Hahn, E.J., Paek, K.Y., 2007. Large-scale cultivation of adventitious roots of *Echinacea purpurea* in airlift bioreactors for the production of cichoric acid, chlorogenic acid and caftaric acid. Biotechnol. Lett. 29, 1179-1182.
- Yin, X., Chen, J., Yuan, W., Lin, Q., Ji, Fang, L., 2012. Preparation and antibacterial activity of Schiff bases from O-carboxymethyl chitosan and para-substituted benzaldehydes, Polym. Bull. 68, 1215–1226.

Chapter 10

In vitro roots as a model system to investigate xanthone biosynthesis at a molecular level

The phytochemical analysis performed on wild grown plants showed that roots are accumulation oragns for xanthones. The *in vitro* studies demonstrated that roots are also organs where the synthesis takes place and that xathone biosynthesis can be stimulated by applying external stimuli. Since nothing is known about the expression pattern at tissue or cell level of the gene econding Benzophenone Synthase, the key enzyme in xanthone biosynthesis, root cultures represent a good model system system for cloning and localization of benzophenone synthase transcripts in *in vitro* roots of *Hypericum perforatum* subsp. *angustifolium*.

Chapter 11

Localization of xanthone biosynthesis at the transcript and protein levels in roots of *Hypericum perforatum*

Localization of xanthone biosynthesis at the transcript and protein levels in roots of *Hypericum perforatum*

Noemi Tocci[1,2], Asma K. Belkheir[2], Robert Hänsch[3], Benye Liu[2], Gabriella Pasqua[1], Ludger Beerhues[2]

[1]Department of Environmental Biology, "Sapienza" University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

[2]Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Mendelssohnstraße 1, 38106 Braunschweig, Germany

[3]Institute of Plant Biology, Technische Universität Braunschweig, Humboldtstraße 1, 38106 Braunschweig, Germany

Correspondence

Ludger Beerhues, Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Mendelssohnstraße 1, 38106 Braunschweig, Germany; l.beerhues@tu-bs.de

Gabriella Pasqua, Department of Environmental Biology, "Sapienza" University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy; gabriella.pasqua@uniroma1.it

Abstract

Hypericum perforatum is widely used for medicinal purposes. Its classes of bioactive constituents involve xanthones, which are mainly accumulated in roots. The carbon skeleton of xanthones is formed by benzophenone synthase (BPS). A BPS cDNA was cloned from chitosan-treated root cultures of *H. perforatum* subsp. *angustifolium* and heterologously expressed. The molecular and kinetic properties of the recombinant

enzyme were studied. An antisense riboprobe was derived from the coding sequence and used for whole-mount *in situ* hybridization. BPS transcripts were primarily localized to the first cortical cell layer and, to a lesser extent, to the endodermis. Antibodies against BPS were used for immunofluorescence localization of the enzyme protein in longitudinal and cross sections. Bright fluorescence was consistently observed in the apical meristem, the first cortical cell layer, and the endodermis, indicating cell-type specific biosynthesis of xanthones in *H. perforatum* roots.

Key words: *Hypericum perforatum,* xanthones, benzophenone synthase, *in situ* hybridization, immunofluorescence localization.

1. Introduction

Hypericum perforatum L. (Hypericaceae) is a well-known medicinal plant widely used for the treatment of depression and skin disorders (Linde et al., 2008; Müller et al., 2008). The species is characterized by the presence of a number of bioactive constituents, which are derived from the polyketide metabolism (Beerhues, 2011). The major classes of active compounds are hypericins, hyperforins, flavonoids, and xanthones. The scaffolds of these metabolites are formed by type III polyketide synthase (PKS) enzymes, which are known to catalyze formation of a diversity of natural products by varying the starter substrate, the number of chain extension steps, and the mode of intramolecular cyclization (Austin and Noel, 2003). The PKS enzyme that is involved in formation of xanthones is benzophenone synthase (BPS; Liu et al., 2003). The enzyme

catalyzes the decarboxylative condensation of benzoyl-CoA with three molecules of malonyl-CoA to give 2,4,6-trihydroxybenzophenone, which then is converted to xanthones (Schmidt and Beerhues, 1997; Beerhues and Liu, 2009). BPS activity was first detected in *Centaurium erythraea* cell cultures (Beerhues, 1996). BPS cDNAs then were cloned from *H. androsaemum* and *Garcinia mangostana* and functionally expressed (Liu et al., 2003; Nualkaew et al., 2012). Unlike hypericins, hyperforins, and flavonoids, which occur in the aerial parts of *H. perforatum*, xanthones are primarily present in the roots (Crockett et al., 2011; Tocci et al., 2012). Root cultures of *H. perforatum* subsp. *angustifolium* were found to produce particularly high amounts of xanthones, especially after treatment with chitosan (Tocci et al., 2010, 2011, 2012). Xanthone compounds exhibit a number of pharmacological activities (Fotie and Bohle, 2006).

Little is known about the regulation of xanthone biosynthesis in roots, which is true for both the endogenous and the exogenous regulation. Here, chitosan-treated root cultures of *H. perforatum* subsp. *angustifolium* were used to clone a BPS cDNA, which was functionally expressed in *Escherichia coli*. The coding sequence served to derive a specific antisense probe, which was used for *in situ* hybridization. Furthermore, BPS antibodies were employed to localize the enzyme protein and to gain insight into the spatial regulation.

2. Results

2.1 Molecular cloning of a HpaBPS transcript from chitosan-treated roots

Using a homology-based cloning strategy and the mRNA pool from roots treated for 2 days with chitosan, a full-length cDNA was cloned. The 1188 bp open reading frame (ORF), which was re-amplified using a

proof-reading polymerase, encoded a protein of 395 amino acids with a molecular mass of 43.19 kDa and an isoelectric point of 6.2. The deduced amino acid sequence shared 96% identity with a previously isolated but functionally unconfirmed *H. perforatum* cDNA encoding BPS (ABP49616.1). Furthermore, 96 and 94% identities were observed with BPSs from *H. sampsonii* (AFU52908; Huang et al., 2012) and *H. androsaemum* (Q8SAS8; Liu et al., 2003), respectively. Expression of the *HpaBPS* gene in roots was up-regulated by chitosan treatment, as studied by RT-PCR (Fig. 1). There was a basal transcript amount before elicitation, which was massively increased 48 h after the addition of chitosan. The level of 18S rRNA served as control for equal RNA template amounts.

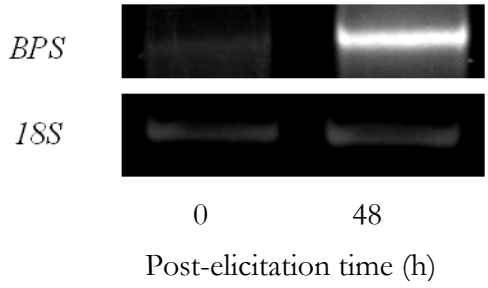


Figure 1. RT-PCR amplification of HpaBPS transcripts (1188 bp) and a 18S rRNA fragment (485 bp) before and after treatment of roots with chitosan.

2.2 Functional Analysis and Characterization

The coding sequence of the HpaBPS cDNA was heterologously expressed in *Escherichia coli* as N-terminally His_6-tagged protein and isolated by affinity chromatography. SDS-PAGE analysis demonstrated a high degree of purity of the 43 kDa protein (Fig. 2).

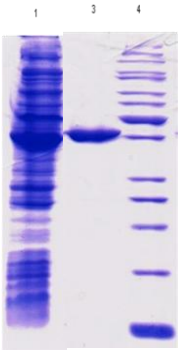


Figure 2. SDS-PAGE analysis of recombinant HpaBPS after staining with Coomassie brilliant blue. Lane 1, total protein from BL21 (DE3) cells harboring the HpaBPS cDNA-containing plasmid after induction with IPTG for 8 h at 25 °C; lane 2, recombinant HpaBPS protein after isolation by Ni^{2+}-chelating chromatography; lane 4, molecular mass markers

The properties of the enzyme were studied. The preferred starter substrate was benzoyl-CoA and the enzymatic product was identified as 2,4,6-trihydroxybenzophenone by liquid chromatography-UV spectroscopy (LC-UV) and liquid chromatography-mass spectrometry (LC-MS) in comparison to a sample of authentic reference compound. A small amount of byproduct, 6-phenyl-4-hydroxy-2-pyrone, was also observed. HpaBPS also accepted to lower extents 3-hydroxybenzoyl-CoA and isovaleryl-CoA. Use of isobutyryl-CoA and butyryl-CoA resulted in the formation of relatively high amounts of the corresponding byproducts. 2- and 4-Hydroxybenzoyl-CoA, acetyl-CoA, and CoA esters of cinnamic acids were not accepted as starter molecules (Table 1). The pH optimum of HpaBPS was 6.5 and the temperature optimum was 35°C. Under the optimum conditions, the kinetic properties of HpaBPS were studied for the preferred starter substrate benzoyl-CoA. The K_m value was 1.992 μM and the k_{cat} value was 7.73 s^{-1},

resulting in a catalytic efficiency (k_{cat}/K_m) of 3,87 s^{-1} μM^{-1}. The K_m value for malonyl-CoA was 12.09. The catalytic efficiency of HpaBPS was similar to those of *H. androsaemum* and *Garcinia mangostana* BPSs (Liu et al., 2003; Huang et al., 2012).

2.3 In situ hybridization of HpaBPS transcripts

Digoxigenin-labeled sense and anti-sense probes were generated and used for in situ localization of HpaBPS transcripts in roots. No specific staining was observed when the sense probe was used for whole-mount in situ hybridization (Fig. 3 A,C). However, intensive staining of both the apical and the distal parts of roots was found upon use of the anti-sense probe (Fig. 3 B,D). In cross sections of roots, no specific labeling was detected after hybridization with the antisense probe (Fig. 4 A). In contrast, the

sense probe localized high levels of HpaBPS transcripts in the elongation zone to the first layer of the cortical parenchyma (Fig. 4 B). In addition, lower transcript levels were found in the endodermis. A similar staining pattern was detected in cross sections of the meristamatic zone (Fig. 4 C). The other cell layers of the cortex were devoid of specific label, as illustrated at higher magnification in Fig. 4 D.

2.4 Immunofluorescence localization of HpaBPS protein

Sections of field-grown roots were incubated with either anti-BPS IgG or pre-immune IgG. No specific fluorescence was observed in longitudinal and in cross sections that were incubated with pre-immune IgG (Fig. 5 a,c). However, when longitudinal sections were incubated with anti-BPS IgG, the apical meristem, the outer

cortical cell layer, and the endodermis were strongly immunofluorescent (Fig. 5 b). The root cortex was relatively thin and consisted of only few cell layers. A similar distribution of fluorescence between the tissues was observed in cross sections (Fig. 5 d). The majority of cells of the first cortical layer and the endodermis immunofluoresced. The exodermis, the inner cortical layers, and the vascular cylinder were devoid of specific fluorescence. The labeling specificity was confirmed by recording the lambda signature.

Table 1. Substrate specificity of HpaBPS based on [2-^{14}C] incorporation

Substrate	Activity (%)	
	Product	Byproduct
Benzoyl-CoA	100[a]	4.5
2-Hydroxybenzoyl-CoA	0	0
3-Hydroxybenzoyl-CoA	43.5	2.3
4-Hydroxybenzoyl-CoA	0	0
Cinnamoyl-CoA	0	0
4-Coumaroyl-CoA	0	0
Acetyl-CoA	0	0
Isobutyryl-CoA	46.4	25.2
Isovaleryl-CoA	11.8	3.7
Butyryl-CoA	36.8	100

[a] Product formation from benzoyl-CoA and malonyl-CoA was set as 100%.

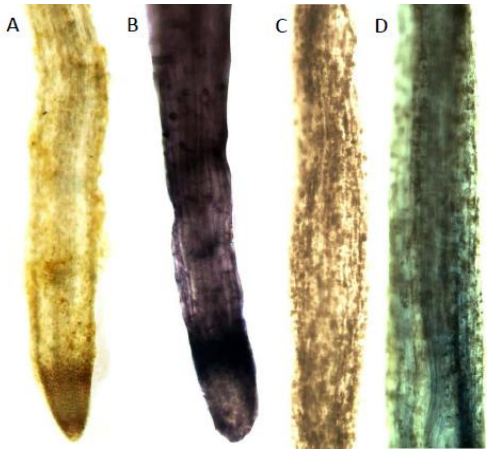


Figure 3. Whole-mount in situ hybridization on apical (A,B) and distal (C,D) regions of *in vitro* grown roots. Samples were treated with either anti-sense (A,C) or sense probes (B,D).

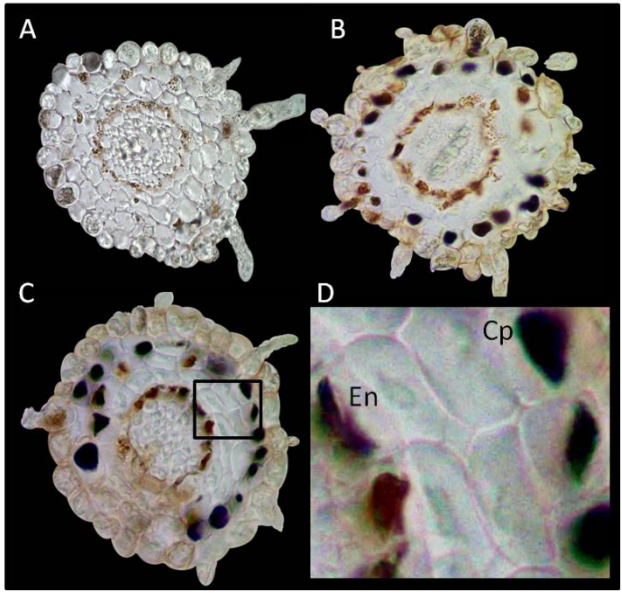


Figure 4. Tissue-specific localization of HpaBPS transcripts in thin sections of roots. A, control section treated with sense probe; B, section from the elongation zone treated with anti-sense probe; C, section from the meristematic region treated with anti-sense probe; D, detail from C showing labeling in the first cortical parenchyma layer (Cp) and the endodermis (En).

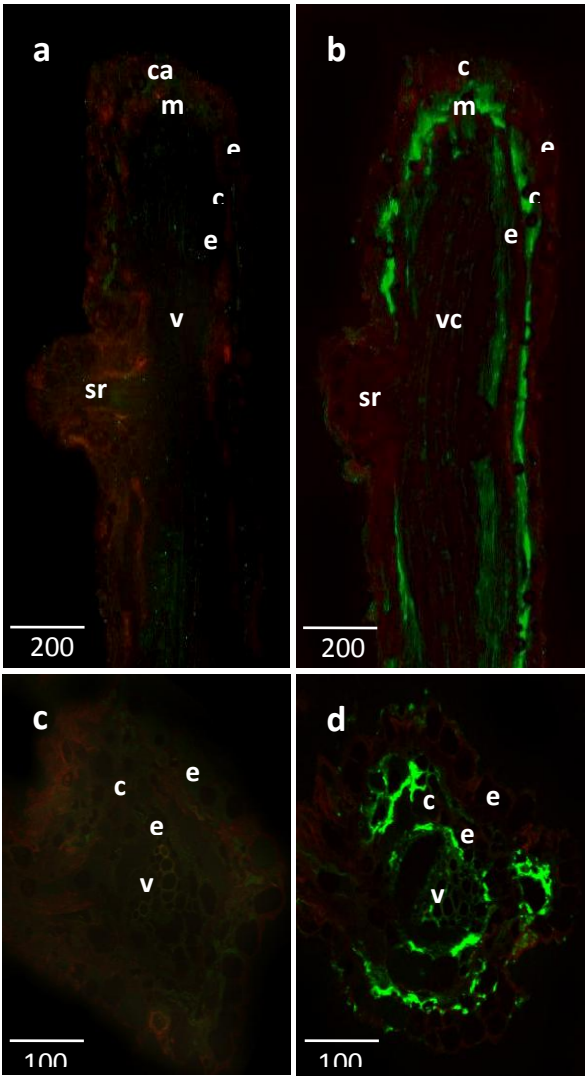


Figure 5. Immunofluorescence localization of HpaBPS protein in roots of field-grown plants. a,b longitudinal sections incubated with pre-immune IgG (a) and anti-BPS-IgG (b); c,d cross-sections incubated with pre-immune IgG (c) and anti-BPS-IgG (d). ca, calyptra; co, cortex; en, endodermis; ex, exodermis; me, meristem; sr, side root; vc, vascular cylinder.

3. Discussion

The present studies of the spatial regulation of *BPS* expression in roots of *H. perforatum* demonstrate cell-type specific biosynthesis of xanthones. The major site of xanthone formation is the first cortical cell layer, as consistently revealed by *in situ* hybridization of BPS transcripts and immunofluorescence localization of the BPS protein. The cortex of *H. perforatum* roots is relatively thin and consists of only a few layers of storage parenchyma cells (Ciccarelli et al., 2001; Maggi et al., 2004). Xanthones appear to function as both preformed defence compounds, so-called phytoanticipins, and pathogen-inducible phytoalexins (Franklin et al., 2009). As phytoanticipins, they protect the roots from underground predators and microbial pathogens. For this reason, it appears reasonable that xanthones are accumulated in the outer cortical cell layer, which is, beside the exodermis, the primary site of attack. The defensive role of xanthones and related compounds was demonstrated (Gronquist et al., 2001).

As phytoalexins, xanthones defend against bacterial and fungal pathogens. In *in vitro* cultures, formation of xanthones is readily inducible by the addition of elicitors (Abd El-Mawla et al., 2001; Conceição et al., 2006; ,Franklin et al., 2009; Tocci et al., 2010, 2011). The antimicrobial activity, especially the antifungal property, of xanthones has been demonstrated (Franklin et al., 2009; Tocci et al., 2012). However, little is known about changes in the spatial regulation of xanthone biosynthesis in roots after the addition of elicitors or the infection by fungi and other predators.

A minor site of xanthone formation is the endodermis. This cell layer, beside the pericycle, has also been implicated in the biosynthesis of tropane and pyrrolizidine alkaloids (De Luca and St Pierre, 2000; Facchini, 2001; Moll et al., 2002). This localization may be related to the translocation of products. The functional aspects of xanthone biosynthesis in endodermis cells remain to be clarified and the expression patterns of further genes involved in this biosynthetic pathway need to be studied. Translocation of intermediates has recently been proposed for hypericin biosynthesis in *H. perforatum* (Zobayed et al., 2006).

4. Materials and Methods

***Chemicals*:** All the CoA esters of cinnamic acids and benzoic acids, apart from benzoyl-CoA, were synthesized as described in the literature (Liu et al., 2003). Malonyl-CoA, acetyl-CoA, and benzoyl-CoA were purchased from Sigma-Aldrich (St Louis, MO, USA). 2,4,6-Trihydroxybenzophenone was obtained from ICN (Meckenheim, Germany). 6-Phenyl-4-hydroxy-*2H*-pyran-2-one (benzoyldiacetic acid lactone) was purchased from Aurora Fine Chemicals (Graz, Austria).

***Plant materials*:** *In vitro* roots were obtained from callus of *H. perforatum* and cultured as described by Tocci et al. (2011) in half-strength Murashige and Skooge (MS; 1964) medium supplemented with 1 mg/L indole-3-butyric acid (IBA). Liquid cultures were established by inoculating 0.02 g dry weight (DW) of roots in 250 ml Erlenmeyer flasks containing 100 ml liquid MS. The flasks were shaken at 100 rpm at 25 ± 1°C and maintained in the dark. *BPS* gene expression was stimulated by adding chitosan (medium molecular weight; Sigma-Aldrich, Milan, Italy)

at a final concentration of 200 mg/l, on day 8 of culture. Root samples were harvested by filtration after 11, 15, 18, and 23 days of culture and subjected to extraction for high-performance liquid chromatography (HPLC) analysis. For RNA extraction, roots were harvested by filtration after 48 h post-treatment.

***Cloning of HpaBPS cDNA*:** mRNA from 48 h chitosan-treated roots was extracted according to the protocol optimized by Jaakola et al. (2001) for flavoinoid-rich plant materials. BPS cDNA was amplified using mRNA from 48 h chitosan-treated roots by RT-PCR and PCR using primers designed according to the sequence of BPS from *Hypericum calycinum*: the forward primer was 5'-GCATGCTAGCATGGCCCCAGCAATGGAATA-3' and the reverse primer was 3'-GCATGGTACCTCACTGGAGGATGGGGA-5'. PCR was carried out using Taq DNA polymerase (Peqlab, Erlangen, Germany). After denaturation at 95°C (2 min), 30 cycles were performed at 95°C (30 sec), 64°C (30 sec), and 72°C (1 min). The final extension was at 72°C for 15 min. Nucleotide sequences were determined from both strands.

***Heterologous expression and enzyme purification*:** The ORF encoding HpaBPS was reamplified by PCR using Pfu DNA polymerase. The amplified DNA was cloned to pRSET B (Invitrogen, Carlsbad, CA, USA) with *Nhe* I/*Kpn* I site. After sequencing of the ORF on both strands, the recombinant plasmids were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, Amsterdam, Netherlands) for overexpression as N-terminally His_6-tagged protein. HpaBPS was purified to homogeneity using Ni-NTA agarose according to the

manufacturer's instructions (Qiagen, Hilden, Germany). The purification efficiency was monitored by SDS-PAGE.

***Enzyme assay and kinetic data determination*:** The standard assay (250 µl) contained 54 µM starter CoA, 324 µM malonyl-CoA, 0.1 M potassium phosphate (pH 7.0) and 2 µg protein. After incubation at 35 °C for 10 min, the enzymatic products were extracted and detected as described previously (Liu et al., 2003). Kinetic constants were determined using eight substrate concentrations covering the range of 0.2-12 K_m, the concentration of the second substrate was saturated.

***Digoxigenin-labelled riboprobe synthesis and whole-mount in situ hybridization*:** Digoxigenin-labelled riboprobes against BPS were generated using the *mir*Vana Probe Construction Kit (Ambion). The antisense probe (CCGAGCCAAACCCTTGGTCGGGAcctgtctc) was designed following the analysis of alignments. The sense probe from the same region was used as a negative control. Whole-mount *in situ* hybridization was performed according to the protocol by Hejátko et al. (2006) on *in vitro* cultured roots collected at day 8 of culture. Signals were detected with alkaline phosphatase-conjugated sheep antidigoxigenin antibody (1:2000; Roche). Colour detection was carried out by incubating roots with a mixture of NBT (0.15 mg/ml) and BCIP (0.075 mg/ml) (Roche) in complete darkness for 2–4 h and subsequent stopping in ethanol. Expression levels were defined by visual inspection.

***Embedding, sectioning, and microscopy*:** After hybridization, roots were embedded in Technovit 7100 (Kulzer GmbH, Germany) resin according to the manufacturer's instructions and cut in 10 µm sections

by using a microtome. Sections were imaged using a camera dhs MicroCam 5.0 attached to a Zeiss Axiostar plus Optical Microscope.

Immunohistochemical Analysis: Using the resin technique, plant tissue was analyzed under a confocal laser scanning microscope. Root material was fixed on ice for 2 h under reduced pressure (vacuum 0.3 mbar) in fixative solution (2% [w/v] formaldehyde, 0.1% [v/v] glutaraldehyde, 0.1% Triton X-100 [w/v] in 0.1 M phosphate buffer, pH 7.2). The samples were washed twice for 10 min with PBS buffer. Water was gradually removed by 30, 50, 70, and 90% aqueous ethanol and finally 3 × 100% ethanol. Each step of incubation took 30 min at room temperature. After dehydration, the plant tissue was embedded in Technovit 7100 (Heraeus-Kulzer, Hanau, Germany) according to the manufacturer's instructions. Fixed tissues were cut into thin segments (3.5 μm) using a microtome (HM 355 S; Microm, Walldorf, Germany). The sections were transferred onto diagnostic microscope slides (Teflon®; Roth, Karlsruhe, Germany) using a drop of distilled water and left to dry. The thin sections were incubated in 50 mM ammonium chloride solution at 37°C for 15 min, washed with water, incubated in 50 mM glycin solution at 37°C for 15 min and washed again with water. The sections were then blocked at 37°C for 30 min with BSA-blocking solution (10% [w/v] BSA and 0.1 % [w/v] fish gelatine in PBS buffer, pH 7.2), washed once with water and 3 × 10 min with PBS buffer. Thereafter, the sections were incubated with either anti-BIS IgG oder preimmune IgG (1:25 to1:100 dilution) with 1% BSA in PBS buffer at 37°C for 1 h. They were washed once with water and 3 × 10 min with PBS buffer. Finally, the sections were incubated in the dark with

fluorescence dye-conjugated goat anti-rabbit antibody as secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG (H+L); Molecular Invitrogen, Darmstadt, Germany) diluted 1:100 with 1% [w/v] BSA in PBS buffer at 37°C for 1 h. After washing 3 × 15 min with PBS buffer and then with water, sections were left in the dark at 37°C until dryness. Mounting medium (Citifluor; Agar Scientific, Essex, UK) was used to adhere the coverslip to the tissue section. The tissues were viewed using a laser scanning microscope (Zeiss LSM 510META, Göttingen, Germany). The scan mode was channel scan. The imaging of whole-mount specimens was recorded using a digital camera (Axioskop 2; Zeiss) and the software Axio Vision 3.0 (Zeiss).

References

- Abd El-Mawla AMA, Schmidt W, Beerhues L (2001) Cinnamic acid is a precursor of benzoic acids in cell cultures of *Hypericum androsaemum* L. but not in cell cultures of *Centaurium erythraea* RAFN. *Planta* 212, 288-293
- Austin MB, Noel JP (2003) The chalcone synthase superfamily of type III polyketide synthases. *Natural Product Reports* 20, 79-110
- Beerhues, L. (1996) Benzophenone synthase from cultured cells of *Centaurium erythraea*. *FEBS Lett.* 383, 264-266
- Beerhues, L. (2011) Biosynthesis of the active Hypericum perforatum constituents. Medicinal and Aromatic Plant Science

and Biotechnology 5: Special Issue 1: Hypericum (Odabas, M.S., Cirak, C., eds.): 70-77

- Beerhues L, Liu B (2009) Biosynthesis of biphenyls and benzophenones – Evolution of benzoic acid-specific type III polyketide synthases in plants. Phytochemistry 70:1719-1727
- Ciccarelli, D., Andreucci, A.C. and Pagni, A. A.(2001): Translucent glands and secretory canals in *Hypericum perforatum* L. (Hypericaceae): Morphological, anatomical and histochemical studies during the course of ontogenesis. *Annals of Botany*, 88: 637-644
- Conceição, L. F. R., Ferreres, F., Tavares, R. M., and Dias, A. C. P., (2006): Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation. *Phytochemistry,* 67: 149–155.
- Crockett, SL, Poller, B, Tabanca, N, Pferschy-Wenzig, EM, Kunert, O, Wedge, DE, Bucar, F (2011) Bioactive xanthones from the roots of *Hypericum perforatum* (common St John's wort), J. Sci. Food Agric. 91, 428-434
- De Luca, V, St Pierre, B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci. 5, 168–173
- Facchini, P.J. (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 29–66

- Fotie, J, Bohle, DS (2006) Pharmacological and biological activities of xanthones, Curr. Med. Chem. 5, 15-31
- Franklin G, Conceição LFR, Kombrink E, Dias ACP (2009) Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. *Phytochemistry* 70, 60-68
- Gronquist M, Bezzerides A, Attygalle A, Meinwald J, Eisner M, Eisner T (2001) Attractive and defensive functions of the ultraviolet pigments of a flower (*Hypericum calycinum*). *PNAS* 98, 13745-13750
- Hejátko, H, Blilou, I, Brewer, PB, Friml, J, Scheres, B, Benková, E (2006) *In situ* hybridization technique for mRNA detection in whole mount *Arabidopsis* samples. *Nature Protocols* 1, 1939-1946
- Jaakola, A.M., Pirttilä, M., Halonen, A. Hohtola (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotech. 19, 201-203
- Linde K, Berner MM, Kriston L (2008) St John's wort for major depression. *Cochrane Database of Systematic Reviews* 4, CD000448
- Liu B, Falkenstein-Paul H, Schmidt W, Beerhues L (2003) Benzophenone synthase and chalcone synthase from *Hypericum androsaemum* cell cultures: cDNA cloning, functional expression, and site-directed mutagenesis of two polyketide synthases. *Plant Journal* 34, 847-855
- Maggi, F., Ferretti, G., Pocceschi, N., Menghini, L. and Ricciutelli, M., (2004): Morphological, histochemical and

phytochemical investigation of the genus *Hypericum* of the Central Italy. *Fitoterapia*, 75: 702-711

- Moll, S., Anke, S., Kahmann, U., Hansch, R., Hartmann, T. and Ober, D. (2002) Cell-specific expression of homospermidine synthase, the entry enzyme of the pyrrolizidine alkaloid pathway in Senecio vernalis, in comparison with its ancestor, deoxyhypusine synthase. Plant Physiol. 130, 47–57
- Müller M, Essin K, Hill K, Beschmann H, Rubant S, Schempp CM, Gollasch M, Boehncke WH, Harteneck C, Müller WE, Leuner K (2008) Specific TRPC6 channel activation, a novel approach to stimulate keratinocyte differentiation. *Journal of Biological Chemistry* 283, 33942-33954
- Murashige, T, Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15, 473-497
- Nualkaew N, Morita H, Shimokawa Y, Kinjo K, Kushiro T, De-Eknamkul W, Ebizuka Y, Abe I. (2012) Benzophenone synthase from Garcinia mangostana L. pericarps. Phytochemistry 77, 60-69
- Schmidt, W, Beerhues, L (1997) Alternative pathways of xanthone biosynthesis in cell cultures of *Hypericum androsaemum* L. FEBBS Lett. 420, 143-146
- Tocci, N, Ferrari, F, Santamaria, AR, Valletta, A, Rovardi, I, Pasqua G (2010) Chitosan enhances xanthone production in

Hypericum perforatum subsp. *angustifolium* cell cultures. Nat. Prod. Res. 24, 286-293

- Tocci, N, Simonetti, G, D'Auria, FD, Panella, S, Palamara, AT, Valletta, A, Pasqua, G (2011) Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity, Appl. Microbiol. Biotechnol. 91, 977-987
- Tocci, N, Simonetti, G, D'Auria, FD, Panella, S, Ferrari, F, Pasqua, G (2012) Chemical composition and antifungal activity of *Hypericum perforatum* subsp *angustifolium* roots from plants collected in field and grown under controlled conditions. Xx
- Zobayed, SMA, Murch, SJ, Rupasinghe, HPV, Saxena PK (2004) *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). Plant Sci. 166, 333-340
- Zobayed SMA, Afreen F, Goto E, Kozai T (2006) Plant-environment interactions: Accumulation of hypericins in dark glands of *Hypericum perforatum*. *Annals of Botany* **98**, 793-804

Chapter 12:
Discussion

H. perforatum is one of the most studied medicinal plants, but little is known about xanthone biosynthesis in the species.

The main topic of the project has been the study of xanthone biosynthesis both in *planta* and in *in vitro* systems of *H. perforatum* subsp. *angustifolium* (Hpa), a rare subspecies showing higher secondary metabolites production in comparison with other *H. perforatum* subspecies (Southwell and Campbell, 1991). The interest in xanthones relies in their documented biological activities (Fotie and Bohle, 2006), which find applications in solutions for human health. Particularly interesting is the antifungal activity exerted by these compounds in the light of the growing demand for antifungals as a consequence of the worldwide increased incidence of fungal infections and of the spread of multidrug-resistant strains of fungi.

The present investigation has been characterized by an approch of both basic and applied research and has been articulated in two main aspects deeply connected:

1. Investigation of xanthone biosynthesis at histochemical, chemical and molecular levels both *in planta* and in *in vitro systems.*
2. Development of root cultures as an *in vitro* system to produce xanthones and xanthone-rich and standardized extracts for applicative purposes in the field of antifungal therapy.

The fungi selected in the present study were *Candida* spp., the main agent responsible for nosocomial fungal infections (Pfaller and Diekema 2010); *C. neoformans*, a common life-threatening human fungal pathogen; and

dermatophytes, which are responsible for fungal skin infections (Seebacher et al. 2008).

Xanthone accumulation in planta

In order to investigate xanthone distribution in wild grown Hpa plants, samples were collected in two sites nearby Rome (Italy) characterized by different altitude and soil composition: Tivoli Terme is at 68 m above the sea level (asl) and is characterized by a calcareous soil; Poli is at 453 m asl and the soil is volcanic. The chemical analysis showed that xanthones are not distributed all over the plant, but their accumulation is confined to the roots. 1,7-dihydroxyxanthone, 5-O-methyl-2deprenylrheediaxanthone B, kielcorin, paxanthone, 1,3,5,6-tetrahydroxyxanthone, and 1,3,6,7- tetrahydroxyxanthone were detected in Hpa. The xanthones 1,7-dihydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone and paxanthone were reported in this study for the first time for *H. perforatum* plants collected in field. Variability has been observed in the xanthone content of the roots collected in the two considered areas (0.43± 0.03 and 0.18±0.02 mg/g DW in Tivoli Terme and Poli, respectively). It is known that secondary metabolite content is a quantitative trait and that levels can be influenced by environment. Although the total xanthone amount is quite low, 1,7-dihydroxyxanthone, and 1,3,5,6- and 1,3,6,7-tetrahydroxyxanthones are reported by other authors for antimicrobial activity (Fotie and Bohle, 2006). In a study by Crockett el al. (2011), the extracts of roots of *H. perforatum* were tested for growth inhibition of plant pathogenic fungi of the genera *Colletotrichum, Botrytis, Fusarium* and *Phomopsis*, and 5-O-methyl-

2-deprenylrheediaxanthone B was found to possess antifungal activity in particular against *Phomopsis*.

In the present study the extract of Hpa roots collected in Tivoli Terme, which was the richest in xanthones, has been tested for the first time against a broad panel of human fungal pathogens and showed a moderate antifungal activity against dermathophytes and *C. neoformans* (geometric MIC mean 215.27 μg/ml and 53.8 μg/ml respectively) .

***Biomolecular studies of xanthone biosynthesis* in planta**

In order to investigate xanthone biosynthesis *in planta* at a biomolecular level, the gene encoding for benzophenone synthase, the key enzyme in xanthone biosynthetic pathway, has been studied. Xanthones have been detected in *H. perforatum* plants (Crockett et al., 2011; Tocci et al., under revision), but nothing is known about the expression pattern of the genes involved in their production. Root cultures of Hpa have been shown to be an *in vitro* system in which the synthesis of xanthones can be regulated by external stimuli. Chitosan and its derivatives were found to be able to induce a massive accumulation of xanthones in *in vitro* cells and roots (Tocci et al., 2010; Tocci et al., under revision) suggesting a rapid induction of xanthone-related gene expression. In the light of the above mentioned data, root cultures treated with chitosan have been studied for cloning and characterizing a full length BPS gene in order to generate a probe for the localization of the relative transcript expression in seedlings. The cloning work led to the obtainment of a 1188 bp ORF which encoding for a 43.19 kDa protein, which deduced aminoacid sequence showed 96% identity with BPS from *H. perforatum* and *H. sampsonii* and 94% with *H. androsaemum* trihydroxybenzophenone

synthase. The RT-PCR showed that the expression of the studied cDNA in roots deeply increased after treatment with chitosan. The coding cDNA was heterologously expressed in *E. coli* and purified to assay the enzyme activity *in vitro*. The functional analysis confirm the identity of the protein as BPS. The substrate specificity assays showed that BPS accepted various substrates, but benzoyl-CoA was the preferred starter.

The present studies of the spatial regulation of *BPS* expression in roots of *H. perforatum* demonstrate cell-type specific biosynthesis of xanthones. The major site of xanthone formation is the first cortical cell layer, as consistently revealed by *in situ* hybridization of BPS transcripts and immunofluorescence localization of the BPS protein. The cortex of *H. perforatum* roots is relatively thin and consists of only a few layers of storage parenchyma cells (Ciccarelli et al., 2001; Maggi et al., 2004). Xanthones appear to function as both preformed defence compounds, so-called phytoanticipins, and pathogen-inducible phytoalexins (Franklin et al., 2009). As phytoanticipins, they protect the roots from underground predators and microbial pathogens. For this reason, it appears reasonable that xanthones are accumulated in the outer cortical cell layer, which is, beside the exodermis, the primary site of attack. The defensive role of xanthones and related compounds have been demonstrated (Gronquist et al., 2001).

As phytoalexins, xanthones defend against bacterial and fungal pathogens. In *in vitro* cultures, formation of xanthones is readily inducible by the addition of elicitors (Abd El-Mawla et al., 2001; Conceição et al., 2006; Franklin et al., 2009; Tocci et al., 2010, 2011). The antimicrobial

activity, especially the antifungal property, of xanthones has been demonstrated (Franklin et al., 2009; Tocci et al., 2012). However, little is known about changes in the spatial regulation of xanthone biosynthesis in roots after the addition of elicitors or the infection by fungi and other predators.

A minor site of xanthone formation is the endodermis. This cell layer, beside the pericycle, has also been implicated in the biosynthesis of tropane and pyrrolizidine alkaloids (De Luca and St Pierre, 2000; Facchini, 2001; Moll et al., 2002). This localization may be related to the translocation of products. The functional aspects of xanthone biosynthesis in endodermis cells remain to be clarified and the expression patterns of further genes involved in this biosynthetic pathway need to be studied. Translocation of intermediates has recently been proposed for hypericin biosynthesis in *H. perforatum* (Zobayed et al., 2006). This is the first study of organ localization of *bps* expression in Hpa. Further studies are in progress to localize the cell-specific expression of *BPS* gene in thin sections of *H. perforatum* plant organ at different developmental stages and under elicitation treatments.

Xanthone production in* in vitro *systems

In vitro cultures are a good system to overcome the problems linked to the variability and the seasonal dependence of metabolite recovery from wild grown plants. Cell cultures of *H. perforatum* have been already established by many authors (Dias et al., 2000; Pasqua et al.,2003; Ferrari et al., 2005; Tocci et al., 2010), but the production of xanthones was not exploitable for applicative purposes.

In the present study, cultures of seedlings and *in vitro* regenerated roots have been established and for the first time evaluated for xanthone content.

In comparison with the plants collected in field, chemical analysis showed that roots from *in vitro* plantlets were 27 folds richer in xanthones. Differently, from the roots of wild grown plants, roots of *in vitro* seedling contained toxyloxanthone B, yet they did not contain kielcorin.

A comparison of antifungal activity against *C. albicans*, non *albicans Candida* species, *C. neoformans*, and dermatophytes of *H. perforatum* roots from wild grown plants and from plantlets grown under controlled conditions highlighted that the geometric mean MIC for the root extracts from the *in vitro* plantlets was 9 times lower for *C. albicans* and for the non-*albicans Candida* species, and 2 times lower for dermatophytes. In parallel it was shown that the extracts deriving from plantlet roots showed an improved antifungal activity in particular against *Candida* spp., highlighting a linear positive correlation between xanthone amount and bioactivity exerted. Although the extracts from roots of *in vitro* seedlings showed interesting antifungal activity, the entire process to get the extracts requires long time (seed germination, plantlets grown, obtainment of great biomass amount, processing of the biomass to separate roots and aerial parts by hand, exsiccation of the plant material, extraction). Moreover, the hardness of automation of the process, in particular to separate the aerial parts from the roots, makes hard the scale-up.

The large-scale cultivation of roots for producing important phytochemicals has been achieved for medicinal plants such as *Nothapodytes foetida* (Fulzele et al. 2002), *Panax notoginseng* (Gao et al. 2005), and *Echinacea purpurea* (Wu et al. 2007). Although root cultures of *H. perforatum* have also been established by other authors (Cui et al. 2010; Goel et al. 2009), the capacity of these cultures to produce xanthones has never been explored. In the present work, cultures of *in vitro* regenerated Hpa roots have been shown to be a good alternative biotechnological system to produce xanthone-rich extracts. Culture conditions for root biomass increase and xanthone production have been optimized and our data indicated that a concentration of 1 mg l^{-1} IBA in the culture medium (Medium 1) was the most suitable to obtain not callused roots and high production of xanthones. The highest concentrations of IBA inhibited xanthone accumulation, confirming reports that high auxin levels are often deleterious to secondary metabolites in other species (Dornenburg and Knorr 1995; Chan et al. 2005). Cultured roots produced xanthones in large amounts and at higher concentration in comparison with roots from seedlings. Moreover the extracts showed a very stable trend of xanthone accumulation throughout the culture period. Elicitation treatments, revealed that xanthone accumulation can been stimulated by chitosan, a polymer that is able to influence biosynthesis of xanthones in cell cultures of Hpa (Tocci et al. 2010). Chitosan treatment resulted in a cessation in growth and a decrease in biomass. Moreover, the elicitation induced a high, even if transient, xanthone production with a peak of 14.26±0.627 mg/g DW after 7 days of treatment. Regarding the antifungal activity of the extracts, an

encouraging results was observed for all of the fungal strains tested: *Candida* spp., *C. neoformans*, and dermatophytes. This activity significantly increased when using chitosan, and high activity was found for extracts obtained after 7 days of treatment (mean MIC of 83.4 μg ml^{-1}, 39.1 μg ml^{-1} and 114 μg ml^{-1} against, respectively, *Candida* spp, *C. neoformans*, and dermatophytes), confirming the positive linear correlation between xanthone content and antifungal activity. Xanthones therefore could be the principal constituents responsible for the antifungal activity.

In the light of the reported observations, a further stimulation of xanthone production was induced by subculturing roots in a medium composed by a combination of growth regulators previously optimized for cell cultures of Hpa (Tocci et al., 2010). The medium contained 1-naphthalenacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Medium 2). Previous studies have shown that NAA stimulated xanthone accumulation in cell suspension cultures of *Hypericum perforatum* (Dias et al., 2001; Tocci et al., 2010). The roots cultures in this conditions were able to produce 3.53 times higher xanthone production and showed from 5 to 10 times higher antifungal activity against *Candida* spp., *C. neoformans,* and dermatophytes, but the biomass growth was negatively affected.

To combine the collected knowledge a three-step culture system was developed to optimize the culture conditions of *in vitro* roots in order to obtain the best xanthone production. The system consisted in a first step culturing roots in Medium 1 to obtain a consistent amount of root biomass; a second step of subculture in Medium 2 to increase secondary metabolite production and a third step with the addition of chitosan to

further stimulate xanthone production. The extracts obtained from roots cultured in Medium 2 and treated with chitosan were the richest in xanthones compared to the previous samples and showed an improved antifungal activity against all the tested strains of *Candida* spp., *C. neoformans,* and dermatophytes.

Chitosan has been shown to stimulate xanthone biosynthesis, but in spite of its advantages, the poor solubility, low surface area, and porosity are the major limiting factors in its utilization for applicative purposes. Chemical modifications of chitosan are increasingly studied for the potential of providing new applications. O-carboxymethylchitosan (CMC) is a water-soluble chitosan derivative, which has been widely studied because of ease synthesis, ampholytic character and possibilities of numerous applications and very low toxicity (Mourya et al., 2010, De Oliveira et al., 2012). CMC has shown to enhance chilling tolerance in cucumber seedlings (Sun et al., 2004) and storage protein accumulation in maize seeds (Osuji et al., 1992) and to delay powdery mildew desease in strawberry (Lowe et al., 2012). To our knowledge this is the first time that the CMC and its derivatives are used as elicitors of bioactive metabolites in *H. perforatum*. In all the analyzed treated samples it has been found that CMC derivatives stimulated the synthesis of xanthones. In comparison with the others, CMC- and N-benzyl-O-carboxymethylchitosan (BzCMC)-treated roots were characterized by the higher amount of xanthones (26.4±2.13 and 9.08±0.16 mg/g DW respectively). CMC, in particular, induced the highest xanthone accumulation even in comparison with commercial chitosan. Extracts of CMC- and BzCMC-treated roots were tested for antifungal activity

against *Candida* species, dermatophytes and *C. neoformans* and the best activity was found in all the assayed strains for CMC-treated roots. Bioassay-guided fractionation of extracts from roots treated with CMC led to the individuation of an fraction obtained by biomass extraction with ethylacetate and containing the higher amount of xanthones (6.8%). The fraction showed the best antifungal activity in comparison with the other tested ones. Ethylacetate fraction has been then subjected to chromatographic separation leading to the purification of a bioactive xanthone, the biyouxanthone D, a prenylated xanthone isolated by Tanaka et al. (2010) from roots of *H. chinense* and reported for the first time in the present paper for *H. perforatum*. This xanthone represented the 37.98% of the all detected xanthones in the total extract and the 44.59 % of the ethylacetate fraction. The antifungal activity of biyouxanthone D, reported for the first time in the present work, showed a high activity against *C. neoformans* and dermatophytes. Moreover, in all the tested extracts, a linear positive correlation between antifungal activity and concentration of biyouxanthone D was evident, suggesting that biyouxanthone D is an active constituent of the *H. perforatum* regenerated roots, against dermatophytes and *C. neoformans*.

Little information is available on mechanism of action of xanthones as antifungals, recently Pinto et al (2011) reported that oxygenated xanthones reduced the biosynthesis of ergosterol. Moreover, it is important to underline that biyouxanthone D is a prenylated compound. It is known that the presence of isoprenoid chains is a major determinant of the bioactivity which resides in a enhanced interaction with biological

membranes and in an increased affinity for target proteins (Botta et al., 2006).

The present study represents a step forward in the preparation of formulations containing *H. perforatum* root extracts for applicative uses in the developing of new antifungal agents.

The process is actually under scale-up for the culture in bioreactor in collaboration with the ROOtec bioactives AG company in Witterswil, Swiss.

References

- Chan LK, Dewi PR, Boey PL (2005) Effect of plant growth regulators on regeneration of plantlets from bud cultures of *Cymbopogon nardus* L. and the detection of essential oils from the *in vitro* plantlets. J Plant Biol 48: 142-145. doi: 10.1007/BF03030574
- Cui XH, Chakrabarty D, Lee AJ, Paek KY. Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. Bioresour Techonol, 2010, 101:4708-4716.
- Dias ACP, Seabra RM, Andrade PB, Ferreres F, Ferreira MF, Xanthone production in calli and suspended cells of *Hypericum perforatum*, J Plant Physiol 158 (2001) 821-827.
- Dornemburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures.

Enzym Microb Tech 17:674-684. doi:10.1016/0141-0229(94)00108-4

- Fotie J, Bohle DS. Pharmacological and biological activities of xanthones. Antinfective Agents Med Chem, 2006, 5: 15-31.
- Gao X, Zhu C, Jia W, Gao W, Qiu M, Zhang Y, Xiao P, Induction and characterization of adventitious roots directly from the explants of *Panax notoginseng*. Biotechnol Lett, 2005, 27:1771−1775.
- Goel MK, Kulreja AK, Bishut NS, *In vitro* manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. Plant Cell Tiss Organ Cult, 2009, 96: 1-9.
- Pfaller MA, Diekema DJ. 2010. Epidemiology of invasive mycoses in North America. Cri Rev Microbiol 36:1–53.
- Seebacher C, Bouchara JP, Mignon B (2008) Updates on the epidemiology of dermatophyte infections. Mycopathology 166:335–352.
- Southwell IA, Campbell MH. Hypericin content variation in *Hypericum perforatum* in Australia. Phytochem, 1991, 30:475-478.
- Tocci N, D'Auria FD, Simonetti G, Panella S, Palamara AT, Pasqua G. A three-step culture system to increase the xanthone production and antifungal activity of Hypericum perforatum subsp. angustifolium in vitro roots. Plant Physiol Biochem 57: 54-58.

- Tocci N, Ferrari F, Santamaria AR, Valletta A, Pasqua G, Chitosan enhances xanthone production in *Hypericum perforatum* subsp. *angustifolium* cell cultures. Nat Prod Res 24 (2010) 286-93.
- Tocci N, Simonetti G, D'Auria FD, Panella S, Palamara AT, Valletta A, Pasqua G. Root cultures of *Hypericum perforatum* subsp. *angustifolium* elicited with chitosan and production of xanthone-rich extracts with antifungal activity. Appl Microbiol Biotechnol, 2011, 91: 977-987.
- Wu CH, Dewir YH, Hahn EJ, Paek KY. Optimization of culturing conditions for the production of biomass andphenolics from adventitious roots of *Echinacea angustifolia*. J Plant Biol, 2006, 49: 193−199.